

How to lobby for more science

The future health of science in the United States may depend more directly on the support of public education than on the continuation of the past decade's generous support of basic research.

Dr Frank Press, the president of the National Academy of Sciences in the United States, plainly wishes he were back in the Old Executive Offices in Washington — a building shaped like a wedding-cake, but painted battleship-grey — where he spent some time in another age as President Jimmy Carter's Science Advisor. His stirring address last week to the annual meeting of the academy (*Nature* 338, 693; 1989) must be read in that context. The appointment in the meantime of Dr Allan T. Bromley of Yale as his successor (Keyworth and Graham in between) was no doubt just a piquant coincidence.

Last year, on the same occasion, Press asked that the scientific community should learn to make up its mind what it wants from government. Superconducting Super Collider or space shuttle, high- T_c superconductivity or the human genome? Better that the scientific community should decide for itself than leave the issues in the lap of Congress. Nothing much has happened in response. This year, Press's message was quieter but more urgent: let there be a decent system of public education, double public spending on basic science and be prepared to wait for the benefits (which cannot come quickly).

Press also made a telling administrative point: why cannot the new US president, Mr George Bush, "reform and integrate the incoherent and uncoordinated policies of the federal departments he has inherited"? He meant not merely that policy on research and development should be better run, but that the US government should find a better way of marrying its commendably high expectations of science and technology with its immediate demands of them. (Make SDI work, stop death, cure the trade imbalance by beating Japan at silicon chips, and all that jazz.) Press, with his experience, knows why the task must be virtually impossible even for a president of the United States. Governments of all stripes everywhere are so used to crises (and crisis-management) that they have unlearned how to manage problems with a longer time-scale. The US government's investment in basic research over the past decade, unthinking though it may have been, has been one of the outstanding exceptions to the rule that short-term considerations matter most.

Now, in the United States, there are undoubtedly long-term problems of research equipment and facilities, as Press averred. But the problem that matters most for science, technology and the well-being of US society is the state of public education. Press modestly, if properly,

asks that the research community should campaign for a better image of science as a career, so that more young people will be attracted to it, and for government support for graduate students. But should not the world's best-heeled and most productive academic community be campaigning on a broader front?

Science is most excellent and technology most beneficial not when they are cultivated in isolation but when they are embedded in a generally enlivened culture. Then, operationally, the pool of those who may wish to choose careers in science is enlarged. Yet the state of the public schools in the United States is such that there is a danger that the next generation of scientists, not to mention intellectuals of other kinds, will be drawn from the sons and daughters of the present middle classes. Can that be wise? Should not a campaign be conducted in the United States to ensure that black or Spanish-speaking kids in the urban ghettos — and white kids in the endless rural deserts of North America — learn to read and write and get to use the calculus? Press rightly asks that government policies should be more coherent, but good government is more than good organization. Governments must also choose. This US government, in the interests of the United States and the rest of us, must choose public education. President Bush has said some of the right things and Press has done his bit. Can Bromley help? □

More market force

The British government is devising a market in higher education, but customers and contractors are proxies.

BRITISH academic life continues in upheaval, now (predictably, see *Nature* 338, 445; 1989) because of the government's proposal to increase the tuition fees it pays on behalf of students in higher education. Nominally, the change is a book-keeping transaction. The cost of paying the extra fees (channelled through local education authorities) will be subtracted from the general subventions of higher education, channelled now through bodies inelegantly called funding councils. But in the long run, the effect will be profound. For British universities and colleges, hard pressed enough at present to recruit students, will find themselves competing even more fiercely for young people, who are becoming scarcer all the time for



demographic reasons.

The proposals are nevertheless welcome. On the principle that there is nothing more important to an academic institution than its freedom to determine its own affairs, any increase in the proportion of its income earned by its prime function — educating students — must be applauded. Doubts on that score should be exorcised by the recollection that, in 1981, Sir Keith (now Lord) Joseph, when Secretary of State for Education and Science, halved the level of university fees so as to make institutions more responsive to centrally imposed limits on student numbers. The reversal of that policy is an important change in the government's thinking. Belatedly, the present government has recognized that Britain is educating too small a proportion of its population to the standards that the modern world requires. Academic freedom may have been a secondary consideration, but should never be sniffed at.

The British government's specific proposal is that tuition fees should be increased from £607 a year to £1,600 a year from September 1990, meaning that British universities will derive more than 20 per cent of their income that way. At polytechnics, the proportion will exceed 25 per cent. The number £1,600 has not been plucked from the air, but chosen to be less than the average annual cost of teaching students on the cheapest courses at the most economical institutions. That means that the new funding councils will still be able decisively to affect the shape of higher education by their distribution of general support. But £1,600 is much greater than the marginal cost of teaching students (which cannot differ much from zero), giving institutions a financial incentive to take more students. Among them, there will be some grabbing of each other's business, but the key question is whether they can make broaden the market for higher education and at the same time create the diversity of higher education that Britain sadly lacks.

There is more to come. The government's consultation paper also canvasses the more complicated proposal that, from 1991, tuition fees paid from public funds will vary with the types of courses students elect to follow. At present prices, a humanities student would be worth £1,600, a mathematics student £2,000, one in science or engineering £2,400 and a medical student no less than £3,200. The intention is said to be to make the market for higher education more efficient, but this scheme would help to correct an untoward effect of the flat £1,600 fee, that expansion would be more rewarding in the arts and humanities than expansion in science and engineering, which the government, appearances apart, wishes to see prosper.

There are two snags, one practical and political, one philosophical. If the British government goes along this road and also carries through its equally rational plan for making its subventions of teaching and research explicit, the time will come when the cost of supporting teaching from the public purse will be virtually nothing at some institutions and very large at others. Then all comparisons

will be invidious, and institutions whose teaching is now good, but expensive, will be cheapened — in this connection, made cheaper. The philosophical question is different. If there were a truly free market in higher education, young people would be putting themselves in the hands of institutions offering what they sought and, in return for fees of some kind, would be acquiring rights to be educated as they thought best. That is why most genuinely autonomous universities do not discriminate by cost between courses of different kinds. People must be free to choose. But not, it seems, in Britain. □

Political greenhouse

Nuclear power might help combat the greenhouse effect, but only if governments are more courageous.

INDEFATIGABLE Mrs Margaret Thatcher's conversion last year to environmental causes seems to have stuck. Last week, she organized a private seminar on the subject at which other ministers and senior government officials, including heads of research councils, were given an up-to-date account of where matters now stand, and were encouraged to speculate on what should happen next (see page 7). By all accounts, the meeting went a long way towards the view that the construction of nuclear power stations is essential to abate the accumulation of carbon dioxide in the atmosphere. Nobody will be surprised, but how is the goal to be accomplished?

Chernobyl notwithstanding, there is now an ample historical record to show that nuclear power stations can be built and operated safely. The experience of Western Europe in this regard is quite remarkable. There are also incidents, such as that at the Three Mile Island power station in Pennsylvania, which show that nuclear accidents need not be catastrophic. But most people, for reasons that are entirely understandable, read recent experience differently. Why cannot what happened near Kiev three years ago happen anywhere? And what is to be done with waste?

If the world is the rational place that we pretend, there is no reason why these anxieties should not be laid to rest. The most urgent need is assurance that human errors of the kind responsible for most known accidents will not recur, which argues for an unprecedented degree of openness about the management of nuclear plants. Will governments comply? Waste disposal, which should be a lesser anxiety, has been allowed by all governments' pusillanimity to grow into a monster. The British government has been especially wayward in its careful calculations of the political disadvantages of particular disposal sites. Will it and others now be more courageous?

The diagnosis is correct that nuclear power (together with energy efficiency) could help to avoid or postpone the greenhouse effect. But only governments can now create the climate of *glasnost* in which these benefits can be won. That will not be an easy task. □

MISCONDUCT INQUIRY

Disputed paper still causing problems

- NIH re-opens Baltimore case
- Dingell inquiry alarms researchers

Washington

IN a surprise move, the National Institutes of Health (NIH) last week decided to re-open its inquiry about a disputed paper published in *Cell* in 1986. The decision comes on the eve of two congressional hearings — one scheduled for today and the other for next Tuesday — on the handling of misconduct investigations in science, with a particular emphasis on the *Cell* paper.

Intense interest mixed with apprehension has attended the plans of the House of Representatives energy and commerce subcommittee on oversight and investigation to continue its investigation into the *Cell* paper. The Secret Service is expected to testify at today's hearings about forensic tests performed on the laboratory notes of Thereza Imanishi-Kari, the paper's principal author, and rumours have been rampant that this testimony will reveal some unacceptable tampering.

But Nobel laureate David Baltimore is certain to be the star witness at the hearings. Baltimore, director of the Whitehead Institute at Cambridge, has acted as spokesman for questions about the disputed paper (*Cell* 45, 247: 1986) of which he was a co-author with David Weaver, Moema Reis, Christopher Albanese and Imanishi-Kari, all then of Massachusetts Institute of Technology (MIT), and Frank Costantini of Columbia University. Imanishi-Kari is now at Tufts University.

Colleagues of Baltimore are rallying to his support, claiming that a scientific argument is turning into a witch hunt.

Phillip Sharp of MIT has sent out a "Dear Colleague" letter asking for help in countering the activities of the investigations subcommittee. Sharp says in his letter that the committee "has decided to hassle David and other authors and this has serious implications for all of us". He added in an interview that draconian investigations of laboratory research "will destroy science in this country".

An NIH review panel last year cleared Baltimore and his colleagues of allegations of misconduct (see *Nature* 336, 505; 1988) and recommended that the matter be settled with a note of correction to *Cell*. NIH director James Wyngaarden says the decision to reopen the inquiry came after a series of specific criticisms of the paper were made in letters from Margot O'Toole, the postdoctoral student in the laboratory of Imanishi-Kari who began the controversy over the paper's validity in 1986.

O'Toole's letters point out that not all the data on which the paper was based could be found in laboratory notebooks supplied to NIH's review panel by the paper's authors. The three-member review panel, made up of Joseph Davie of Searle Pharmaceuticals, Hugh McDevitt of Stanford University and Ursula Storb of the University of Chicago, had checked the data on which the paper was based, according to Wyngaarden, but had not carried out a "complete audit point by point". Wyngaarden says he was "not satisfied with the answers" he had received to preliminary inquiries into the newly discovered discrepancy and had decided to re-open the inquiry with "a more careful data audit".

Behind Wyngaarden's careful words lie the worry that the congressional inquiry may seize on errors in the paper as evidence of serious misconduct and attack the ability of scientists to police their colleagues. Congressional subcommittee chairman John Dingell has already pointed out that he regards NIH's performance in the Baltimore case as a "crucial test of their ability to deal with cases of questioned science" (see *Nature* 337, 490; 1989).

But few scientists have been prepared for how far he would go in his investigation. Files and letters have been requisitioned by the committee from authors of the *Cell* paper in a manner more reminiscent of an attack on an organized crime syndicate than a check on two graphs and a table in a three-year-old scientific paper.

That the quarrel over the paper has become so important is largely due to the activity of Walter Stewart and Ned Feder, two NIH scientists well known for their analyses of scientific misconduct. O'Toole raised questions about the paper's validity soon after it was published. But two informal inquiries, neither of which examined the raw data on which the paper was based, failed to find evidence of serious error. The matter might have ended there if Stewart and Feder had not become involved and circulated a controversial analysis of the *Cell* paper, partly based on 17 pages of a notebook allegedly from Imanishi-Kari's laboratory.

The analysis helped to fire two congressional hearings on scientific misconduct in April 1988. Stewart then became a temporary member of the Dingell subcommittee's staff, working on the Baltimore case. Baltimore did not have an opportunity to defend himself at

GENOME SEQUENCING

HUGO to go international

Washington

THE one-year-old Human Genome Organization (HUGO), meeting last week at Cold Spring Harbor Laboratory in New York, is to open three regional offices: at Osaka University in Japan under Kenichi Matsubara, at the Imperial Cancer Research Fund in London under Sir Walter Bodmer, and at the Howard Hughes Medical Institute on the campus of the US National Institutes of Health.

HUGO President Victor McKusick says that the organization will add a mouse genome committee to those on physical mapping and databases, and will structure the organization much like a national academy of sciences, where potential members must be nominated and voted upon. HUGO now has 220 members from 23 countries. Elections will be held later this year to constitute an 18-member governing council.

Carol Ezzell

VENUS PROBE

May 'window' still the target

Washington

THE Magellan Venus radar mapping spacecraft was 31 seconds away from launch last Friday, 28 April, aboard the space shuttle Atlantis when a pump in the shuttle's main engine cooling system failed, forcing postponement. The launch has been rescheduled for today, at 1.48 pm Eastern Daylight Time. It is critical that Magellan be launched before 28 May. After that the alignment of the planets will no longer permit the spacecraft to reach Venus, and the mission — already much postponed — will have to be delayed for at least 18 months.

Joseph Palca

either hearing. The NIH review panel was set up in June.

By the time that panel had presented its draft report in November, Baltimore had already published a letter in *Cell* (55, 541; 1988) that corrected three "misstatements" in his original paper (*Nature* 336, 295; 1988). But the NIH panel was not satisfied. While it cleared Baltimore and colleagues of misconduct or fraud, it agreed with many of O'Toole's original criticisms of the paper and asked for a further letter of correction.

That letter will be soon be published in *Cell*, according to a Whitehead spokesman. But with the NIH inquiry reopened and the congressional hearings imminent, it is now anyone's guess as to how the affair will end. O'Toole, who says she left her career in science as a result of the reaction to her complaints, still says the full truth has not come out.

Alun Anderson & Joseph Palca

More than scepticism

Baltimore

As researchers from laboratories and universities throughout the United States lined up to announce their failure to achieve cold fusion, a late-night session of the American Physical Society (APS), in Baltimore last Monday, took on the atmosphere of a hanging party lacking only its intended victims — Stanley Pons and Martin Fleischmann.

Loud applause greeted the remark by Steven Koonin, of the University of California at Santa Barbara, that the heat supposedly generated in a palladium electrode was a sign of "incompetence, perhaps delusion" on the part of the University of Utah researchers.

More significant than the list of negative results were the explanations offered by some of the speakers for the disputed claims. The 2.2 MeV gamma-rays offered by Pons and Fleischmann as evidence for the production of fast neutrons could, some speakers suggested, be due to radioactive decays of bismuth derived from naturally occurring radon, and the presence of helium-4 in a few parts per million could result from contamination by laboratory air rather than fusion of deuterium.

But the centrepiece of Pons and Fleischmann's claim, that heat is produced in their electrolytic cells in amounts too large to be explained by purely chemical processes, was dissected by Nathan Lewis, of the California Institute of Technology, and W. Meyerhof, of Stanford University, who ascribed the energy generation to poor calorimetry and an inadequate accounting of the data.

By contrast, Steven Jones, of Brigham Young University, met with a polite but generally sceptical reception. His claim of nuclear fusion, signified by neutron emission at a very low rate, survived the evening mostly unscathed, because most of the experimenters reporting their results did not have neutron detectors of the same sensitivity. But J. Dickens, of Oak Ridge National Laboratory in Tennessee, put in a vote against Jones, saying that his own group had failed to find neutrons at a level nearly ten times below what the Brigham Young group has argued for.

A certain amount of enmity had been generated the previous week, when Pons and Fleischmann told the House Committee on Science, Space and Technology on 26 April that they were "sure as sure can be" that cold fusion worked. Chase Peterson, president of the University of Utah, suggested \$25 million as a reasonable amount of money for the federal government to spend on a scaling-up of bench-top fusion experiments.

At the same hearing, Jones made it clear that, although he believed in the reality of cold fusion, he saw it as an inter-

esting piece of physics, not as a technology for energy production. By the time physicists such as Harold Furth, director of the Princeton University Plasma Physics Laboratory, were giving their sceptical testimony, most of the congressmen had left. The hearing nevertheless provided an opportunity for some committee members to praise the virtues of "small science", and applaud the invention of an American technology that will "change the face of the Earth".

But questions about the lack of confirmation by other scientists and the continued absence of a full published account of the work made several congressmen nervous of jumping in too soon with federal funds. To dispel such doubts, Ira Magaziner, a consultant to the University of Utah, listed half a dozen previous US inventions which had been brought to commercial fruition by the Japanese, and urged the committee to move now, even before the science was widely accepted.

Yet it is unlikely that any congressional initiatives will emerge while the scientific status of cold fusion changes daily. Robert Huggins, a materials scientist at Stanford University, was warmly received by the committee for his account of an experiment which seemed to confirm heat generation by cold fusion, but later flew to San Diego and into a more questioning audience when he described his results in full at a hastily organized session of a meeting of the Materials Science Research Society.

Huggins and his colleagues say they have observed temperature differences of up to 10°C between a cell containing deuterated water and an identical cell containing ordinary water, representing an excess energy in the deuterated sample of 15 to 40 per cent.

The secret of his success, Huggins said, lies in the elimination of hydrogen from the palladium electrode and the cell containing heavy water, by repeated melting and recasting of the electrode material and assembly of the apparatus in a glove-box filled with dry nitrogen. Regular hydrogen will readily pollute the experiment, he said, if introduced either as hydrogen occupying sites in the palladium matrix or as water from the atmosphere, which will be absorbed by the heavy water and preferentially hydrolysed at the palladium cathode.

This experiment, as well as the work of Pons and Fleischmann, was criticized at a fundamental level by speakers at the Baltimore meeting last Monday. Both Lewis and Meyerhof spoke of the difficulty of doing exact calorimetry in an open system which gives off gases and needs to be replenished with fresh electrolyte. The rate of energy generation is derived from a

measurement of the temperature of a working cell, and it is in this measurement that experimental niceties can prove overwhelming.

Neither Huggins nor Pons and Fleischmann attempted to stir the electrolyte in their cells, which suggests that temperature gradients can develop, and means that different temperatures can be due either to different energy generation rates or more simply to different placements of the thermometer in the cell.

Both Lewis and Meyerhof had attempted to reproduce the exact cell construction used by Pons and Fleischmann. They did this by examining whatever photographs and diagrams they could find, and deriving a scale from the size of Pons's hand. Both Lewis, by demonstration, and Meyerhof, by calculation, then showed that 'energy generation' of the magnitude claimed could arise because the temperature distribution in the cell was far from uniform, and the thermometer placement gave rise to a misleading heat output.

The problem was aggravated, Lewis said, because the table of results given by Pons and Fleischmann listed energy outputs not as a percentage of the total energy supplied, but as a percentage of the calculated and strictly chemical heat output. The energy discrepancies, as found by Lewis by back-calculation from the published data, are not so startling in magnitude.

In the experiments done by Lewis and his colleagues, the electrolytic cell was stirred to maintain a uniform temperature, and was also kept at a constant temperature by means of a resistive heater: if the electrode voltage was reduced, the heater was turned up by a measured amount to keep the total heat supply the same. Thus the calorimeter had constant internal calibration. In a still-continuing series of experiments and comparisons, Lewis reported that they had found no anomalous heat generation to a level of 6 per cent, the empirically estimated accuracy of their device.

At the end of the session at Baltimore, physicists were left with the comfortable feeling that fusion was dead, except for small effects of the sort claimed by the Brigham Young group. If, and how quickly, chemists come to the same conclusion is not yet apparent.

David Lindley

Corrections

THE statement in our article on 27 April (338, 702; 1989) that the Brigham Young group had not replicated their measurement with H_2O is incorrect. Such a replication is described in their original article (338, 737; 1989). A News story in the 20 April issue of *Nature* (338, 605; 1989) incorrectly refers to a press conference at Texas A & M on 10 March: this should be 10 April. And reports of an experiment at the University of Moscow were received on 12 April, not 12 March. Keith Johnson of MIT was incorrectly referred to as Keith Jones. □

Brilliant but unrealistic?

Washington

THE Strategic Defense Initiative (SDI) takes on a new shape in the defence budget proposals released by the White House last week. Instead of the elaborate system of large interceptor rockets and sophisticated sensor platforms envisaged under the Reagan administration, centre stage is taken by an orbiting swarm of thousands of cheap and simple 'brilliant pebble' interceptor rockets.

Each three-foot-long 'pebble' is guided by an optical sensor linked to a computer and is designed to destroy independently ballistic missiles in their boost phase by slamming into them at high speed.

Presidential backing for brilliant pebbles comes after a year of vigorous lobbying by Edward Teller, former director of the Lawrence Livermore Laboratory and physicist Lowell Wood, director of advanced studies at the same institute. But critics say there is no reason to believe the untried concept is anything more than an intriguing idea. "The current political fad for brilliant pebbles is more a testament to the triumph of hope over reality, political salesmanship and access, and the desperation of SDI programme managers to deploy something sooner rather than later, than it is to any engineering breakthrough", says John Pike, a space policy analyst for the Washington-based Federation of American Scientists.

A key attraction is that the scheme saves money. By concentrating on brilliant pebbles and delaying some other parts of the SDI programme, US President George Bush is able to cut his predecessor's 1990 SDI budget request from

\$5,600 million to \$4,500 million, only a small increase over the amount finally agreed with Congress last year. And much bigger savings may be on the way, according to a report released from outgoing SDI director, Lt General James Abrahamson in February. He claims brilliant pebbles could help halve SDI's estimated costs.

Such considerations weigh heavily as Bush proposes cancelling several military programmes and slowing the development of others, including the B2 Stealth bomber, in order to reduce the defence budget by \$10 thousand million dollars. But Pike points out that "the iron law of aerospace engineering is that everything always looks quick and cheap and simple until you actually start working on it".

Each pebble carries its own sensor and sophisticated computer. Optical sensors are favoured over the infra-red sensors planned for other space-based interceptors because they are cheap and do not require cryogenic cooling. But they may not be efficient. Brilliant pebbles' biggest strength lies in their numbers. As many as 10,000 might be deployed in space making them far less vulnerable to attack than orbiting 'garages' of interceptors. And as each pebble carries its own sensor and could carry out an independent attack there would be less need to worry about some of the complicated control and communications problems that bedevilled earlier versions of SDI. But independent action carries the risk that, once activated, the pebbles would not be brilliant enough to tell friend from foe and would be as likely to shoot down the Space Shuttle as a Soviet rocket.

Alun Anderson

DISARMAMENT

Japan goes international

Tokyo

JAPAN last month hosted a United Nations meeting on disarmament in Kyoto. The meeting, proposed last June by Prime Minister Noboru Takeshita, is the first of its kind to be held in Japan and is indicative of Japan's growing involvement in world affairs. No disarmament agreements were reached — the meeting was intended to be a forum for 'open and frank' discussion — but moves were made towards the establishment of a global seismic network to monitor small underground nuclear explosions.

The Kyoto conference, attended by representatives of 31 countries, covered several aspects of disarmament, including non-proliferation of nuclear, chemical and other weapons, confidence-building measures and nuclear test-ban verification. But particular emphasis was placed on test-ban verification because this is

an area to which Japan can contribute through its expertise in seismology.

Before the conference, scientists attending the meeting saw a demonstration of the exchange of data between Australia and Japan at the Meteorological Agency in Tokyo. Seismologists reported at the working group session on test-ban verification that a global network of 50 seismic stations could detect nuclear blasts as small as one kiloton, and agreements were made among the participants for further exchange of data as a step towards establishing such a network.

The US Pentagon opposes a comprehensive test-ban treaty, insisting that tests are essential for development and modernization of weapons. But a global seismic network could allow a 'step-by-step' reduction in the scale of nuclear tests, as proposed by Japan at the 1984 Geneva Conference.

David Swinbanks

University bombed

Munich

UNKNOWN sympathizers of the terrorist organization RAF (Rote Armee Fraktion) placed firebombs in a hallway of the mathematics institute of the Technical University of Berlin last week, causing several hundred thousand deutsche marks' worth of damage. Police are still searching for the attackers, who spray-painted a message on the wall of the institute expressing support for RAF terrorists held in solitary confinement who had been carrying out a two-month hunger strike.

University Chancellor Ulrich Podewils said that it was not clear why the university was chosen as a target.

S.D.

No museum piece

Munich

CAREER politician Erhard Busek replaced biochemist Hans Tuppy as Science Minister in Austria on 24 April. Busek, 48, has a doctorate in law but has made a name for himself in cultural affairs as deputy mayor of Vienna. Busek, like Tuppy, was nominated by the conservative People's Party (Volkspartei).

In an interview on Austrian television on 24 April, Busek said that he supports his predecessor's goal of investing 1.5 per cent of Austria's gross domestic product in science and technology by 1990. "If we don't do something soon," said Busek, "then Austria will not just have a lot of museums. We will be a museum."

S.D.

Chernobyl censorship

London

SOVIET film-maker Nikolai Mashenko, whose film on the Chernobyl catastrophe was released last October, has made a formal complaint to the Soviet government about the censorship of this work. Whole sequences, he said, were cut at the insistence of the Soviet Ministry of Health and Ministry of Atomic Energy. His complaint was filed several weeks ago, but so far he has received no answer.

V.R.

Danish merger

London

DENMARK'S two largest pharmaceutical companies are to proceed with their planned merger, following shareholder approval last week. Novo-Nordisk, the outcome of the merger between Novo Industri and Nordisk Gentofte, will combine their research on the production of hormones and other polypeptides by recombinant DNA techniques.

The new company will supply close to half of the world's demand for insulin, and continue Novo's position as the largest supplier of industrial enzymes. It will also be increasingly involved in diagnostics, not least as a result of Novo's recent acquisition of two UK companies, IQ (Bio) and Celltech Diagnostics.

P.N.

Two ministries at war

Paris

A PROPOSAL to squeeze defence spending in order to finance an ambitious expansion in education and research, while reducing the balance of payments deficit, has provoked an internal squabble in the French government. The proposal, believed to have come from Prime Minister Michel Rocard, has set the minister for defence, Jean-Pierre Chevènement and finance minister, Pierre Bérégovoy at logger-heads.

The problem has arisen because defence commitments undertaken in 1987 are now turning out to be too heavy in the light of the socialist government's priorities for education — a ticket that helped them win the elections last year. The defence budget growth is traditionally fixed every four years. In 1987, the annual growth rate was set at 6 per cent, with an understanding that the rate for 1990-93 would be revaluated this year. If the 6 per cent increase is continued for the next four years, as was expected, FF470,000 million (\$74,600 million) would be available for a major overhaul of France's military capability agreed in 1987. This includes the construction of a new nuclear-powered aircraft carrier, a combat helicopter and the Rafale fighter plane. But Bérégovoy says the four-year budget cannot exceed FF400,000 million if he is to balance his books while supporting government priorities in education.

Chevènement, himself a former education minister, is adamant that the growth rate in military spending must not fall below 5 per cent up to 1993 if France's security is not to be jeopardised. Even a 4 per cent increase, which he has ruled out outright, would be FF32,000 million more than the defence minister is prepared to pay and would reduce defence spending from its current 3.69 per cent of gross domestic product (GDP) to 3.56 per cent. Education currently receives 4.5 per cent of GDP.

Michel Rocard intervened early last week, saying there is no interministerial conflict, "just normal discussions within a government during a difficult year". In a statement to the press, Rocard added that "there is no question of cutting defence spending, simply a need to discuss the rate of increase". Meanwhile, Chevènement, who is known to have a short fuse, having resigned as education minister in the previous socialist government, is looking to see where cuts can be made. He has already decided to close non-strategic air bases and is considering extending the Rafale construction deadline, even though this could damage French arms export contracts.

Peter Coles

Microbiologist butt of protests

Amherst, Massachusetts

MORE than fifty students were arrested at the University of Massachusetts last week following the occupation of a university building to protest against research supported by the US Army. The target of the protestors was microbiologist Curtis B. Thorne, who studies the anthrax bacillus with funding from the army's biological defence research programme.

As the student occupation went on, a special hearing before the local Board of Health heard testimony on a proposed ordinance to ban from the town of Amherst "the testing, storage, transportation, and disposal of biological materials if funded in full or in any part by the US Army's Biological Defence Research Program". Nearly 200 students, residents, and outside experts drawn from around the region gathered for more than three hours to debate the ordinance which would effectively outlaw Thorne's research from the town limits. A decision is not expected until wider discussion at a town meeting in May.

NIH

Wyngaarden resigns

Washington

THE director of the US National Institutes of Health (NIH), James B. Wyngaarden, announced last week that he will not be continuing in office under the new presidential administration. His resignation will take effect on 1 August.

Although ready to move on to other things, Wyngaarden explained that it was "not so much my decision as theirs". As a Reagan appointee, he was required to offer his resignation on the change of administration. A national search committee headed by James O. Mason, newly appointed Assistant Secretary of Health and Human Services (HHS), will be responsible for nominating potential successors.

During Wyngaarden's seven years in office, the NIH budget has doubled. He has presided over a huge increase in backing for AIDS research and fought successfully to retain control of the human genome project at the NIH. He says that one of his priorities has been to improve the productivity and stability of research. During his tenure, the proportion of five-year awards has risen from 20 to more than 50 per cent.

Wyngaarden has not always toed the White House line. He did not endorse the ban on research using human fetal tissue imposed by the Assistant Secretary for Health last July and he has said publicly that he feels abortion is a matter of private choice. He was not asked about his stance on abortion at his confirmation hearings, but the same may not be true for his successor.

Penelope Austin

University of Massachusetts chancellor Joseph Duffy fought back with a written statement at the meeting vowing to "protect the right of our faculty to conduct any research they choose as long as the scope, methods and results can be fully and freely disclosed", adding that the policy would not be negotiated with students.

Support for the student protests came from several researchers, including Richard P. Novick, molecular biologist and director of the Public Health Research Institute in New York. He said that Thorne's research presented a health threat to the community and "could not be construed as for peaceful purposes".

Thorne denies that his five-year, \$600,000 research contract poses a safety hazard. He says that the issue of safety is not the protesters' real concern, and that his research is being used merely as a tactic to force a debate on "the larger issue" of "whether it's moral or immoral" to accept funding from the military — a debate Thorne says he will not participate in.

Seth Shulman

US ACADEMY

Welcome to the club

Washington

THE US National Academy of Sciences held its 126th annual meeting in Washington last week, where it held symposia, listened to speeches and elected 60 new members. Membership is "one of the highest honours that can be accorded a US scientist or engineer", the academy says. But who has the best chance of being elected by their peers? According to statistics compiled by the academy's own news and public information office, the successful candidate will most likely come from

Academy membership April 1988

University	No. of members
Harvard Univ.	110
Massachusetts Inst. Technology	81
Stanford Univ.	79
Univ. California, Berkeley	82
California Inst. Technology	51
Yale Univ.	51
Univ. California, San Diego	44
Univ. Chicago	41
Univ. Wisconsin, Madison	37
Princeton Univ.	39

Harvard, have won a Nobel prize in physiology or medicine, and be male.

As of last year's elections, 1,486 of the 1,539 (96.6 per cent) living members of the academy were men. Since 1863 when the Academy was established there have been only 65 female members.

One hundred and forty-eight academy members have won Nobel prizes; 111 before they were elected, 29 after, and 6 the same year.

Joseph Palca

GREENHOUSE EFFECT

Thatcher hosts symposium

London

BRITAIN's prime minister, Mrs Margaret Thatcher, demonstrated her resolve to tackle the greenhouse effect by holding a seminar for senior ministers in her home at 10 Downing Street last week. Eight cabinet ministers and 24 scientists and businessmen attended the seminar, which was intended to educate the politicians about the consequences of a global temperature rise and possible measures to prevent it.

Among those present were the heads of the science research councils; the directors of the Meteorological Office, the British Antarctic Survey and the Forestry Commission; the heads of the Central Electricity Generating Board, Shell UK and the Confederation of British Industry; and senior ministers from the departments of energy, environment, education and science, trade and industry, transport, agriculture, health, and for home and foreign affairs.

This unorthodox forum for the discussion of environmental problems seems to illustrate an unusual scientific awareness at the highest levels in any government. Sir George Porter, the president of the Royal Society, said this was "the first time

in history that a head of state has held a scientific symposium in her own home". It is the right way for science to be disseminated through government, and ought to happen more often, he said.

The one-day private discussions began with speakers outlining the problem. Professor Tom Wigley, of the climate research unit at the University of East Anglia, reported climate change predictions. He estimated a global temperature rise of between 1.1 and 1.9°C by 2030, accompanied by a global sea-level rise of 17–26 centimetres.

The government's adviser from the Energy Technology Support Unit, Ken Currie, described measures which could be taken to mitigate the greenhouse effect. Energy efficiency could make the greatest contribution to reducing emissions of greenhouse gases. Switching from coal-fired to gas-fired generation of electricity, increased use of nuclear power and renewable energy technology would also have a significant effect.

Thatcher now plans to take action to tackle the greenhouse effect at a national level and to urge international action, although no details have yet been announced.

Christine McGourty

CORAL REEFS

Protests move an airport

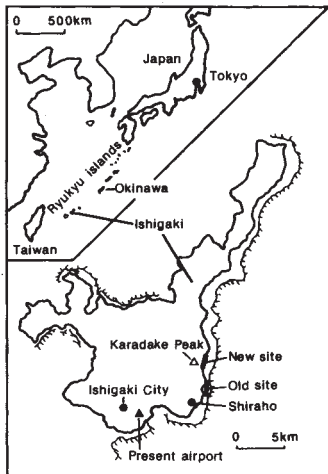
Tokyo

INTERNATIONAL criticism has forced Japan to revise its plans to build an airport on top of a coral reef off Ishigaki, an island at the southern end of the Okinawan chain (see *Nature* 324, 100; 1986). The reef supports extensive stands of a rare species of coral protected by international agreement.

Last week the Environment Agency released a report opposed to the airport plan and within hours the Okinawan Prefectural government, which for years has vehemently resisted opposition to the airport, announced that it was abandoning the site at Shiraho on the east coast of Ishigaki. The Okinawan government then put forward a new proposal to relocate the airport on the reef a few kilometres to the north where the coral is already dead. But conservationists fear that construction at the new site will still pose a threat to the island's living coral — silt from building activities being one danger

— and opposition to the airport seems likely to continue.

The new airport was first proposed more than a decade ago to allow jumbo jetloads of tourists to fly into the island direct from Tokyo (Ishigaki's present airport is too small to handle jumbo jets and local farmers oppose extension of the runway). But, although the majority of islanders support the airport plan, it has



attracted opposition from conservationists around the world because the reef fringing Ishigaki supports extensive stands of the rare Pacific blue coral *Helipora cerulea*, a species listed under the Convention of International Trade in Endangered Species.

In February last year the International Union for Conservation of Nature and Natural Resources adopted a resolution calling on the Japanese government to abandon the plan. Opposition from Japan's marine biologists has been lacking but the Environment Agency, in an unusual move, carried out

RAIN FORESTS

Brazilian general stung by Sting

São Paulo

THE growing debate in Brazil about the devastation of the rain forests had its moments of burlesque last week. While the British rock music star Sting toured Europe accompanied by the Brazilian Indian chieftain Raoni in a campaign to save the rain forest, the Brazilian Army minister fumed. "They are actors playing their roles, with financial advantages for both", said General Leonidas Pires Gonçalves of the two. But the general then went even further in his comments following a visit to Congress, saying that "the culture of the Indians is very low and doesn't merit respect".

In Congress, homage was being paid to representatives of Indian nations: 19 April is officially 'Indian's day' in Brazil.

Brazilian anthropologists responded angrily to the general's comments. They feared that the statements could incite new violence against the country's natives. Darci Ribeiro, anthropologist and ex-vice-governor of Rio de Janeiro State, said that "Marshal Rondon would be ashamed of his army if he listened to the minister's statements". Marshal Candido Mariano da Silva Rondon (1865–1958) was known for his pioneering work in isolated regions of Amazonia and was a protector of Indians. The state of Rondonia was named after him. Ironically, Rondonia is the most heavily deforested region in Amazonia.

Ricardo Bonalume Neto

an environmental impact assessment in the area in November. The assessment, released last week, concludes that the airport runway, to be constructed by infilling the lagoon between the reef and the shore, "cannot be guaranteed to not have a detrimental effect" on the stands of blue coral that are only metres away.

The Okinawan government had no choice but to abandon the site because Environment Agency approval is required for reclamation sites in the sea over 50 hectares in area. But the new proposal cleverly avoids this limit by locating the runway halfway on land and halfway into the lagoon.

Professor Yuji Yonemori of Ryukyu University, Okinawa, who is one of the few Japanese academics to have opposed the airport, welcomed the decision to abandon the site at Shiraho but worries that construction at the new site, only 4 kilometres away, may have a "bad effect".

Many of the fringing coral reefs in the Okinawan islands are already dead. Marine biologists are uncertain of the causes but the most likely culprits are run-off of silt from construction and agricultural projects and invasion of the reefs by the coral-eating starfish *Acanthaster planci*.

David Swinbanks

Academy on the move

Munich

OFFICIALS at the 'Academy of Science and Technology in Berlin' have begun reluctantly to consider moving the academy out of West Berlin. Berlin's new mayor, Walter Momper (Social Democrat), declared in his inaugural speech on 13 April that the academy would be closed "as soon as possible". The academy, a favoured project of the previous Christian Democratic city government, was founded in 1987 as an interdisciplinary think-tank concentrating on technology and society. Academy spokesman Eberhard Vogt said the academy has had offers from three other *Länder* in West Germany but that remaining in Berlin is a "priority".

Several major science organizations in West Germany, including the science advisory council Wissenschaftsrat, the Deutsche Forschungsgemeinschaft and the Max Planck Society, criticized Momper's plan to close the academy as "a politically motivated exertion of influence". S.D.

Independent students

London

HUNGARY'S first independent students' organization, the Students' Chamber, was established at the end of last month in the faculty of economics of the Pannonius University of Pecs. The chamber, set up following the voluntary dissolution of the faculty committee of the Communist Youth Union (KISZ), is essentially non-political and "free of ideologies". The purpose of the chamber, the founding document says, is the representation and protection of students' interests. The only political question on which the students' chamber will take a stance, it says, are those directly connected with higher education. V.R.

Call for papers

Sydney

Two University of Queensland reports agree that there is a correlation between the number of publications a scientist produces and the chance of obtaining funds.

Anthony Wheeler, a senior tutor in the Department of Pharmacology and Physiology, claims that scientists are forced to boost the numbers of their publications as competition heightens for declining budgets. But increases in numbers, Wheeler writes, are often produced by publishing the same material repeatedly, the division of one set of results to produce as many separate short publications as editors will bear (referred to as 'salami science') and increasing the numbers of co-authors on each paper.

Peter Sheehan, the university's Pro-Vice Chancellor of Research, has looked at indicators of research performance and finds that "the number of papers published did relate closely to the amount of money and grants given". T.E.

Human rights assessed

London

MANDATORY testing for human immunodeficiency virus (HIV) or the compulsory registration of suspected HIV carriers could lead to prosecution under international human rights law, according to a report published recently by the British Medical Association.

The report, written by Paul Sieghart, a lawyer specializing in human rights, examines the legitimacy, in terms of the international law of human rights, of the policies which governments might adopt in order to limit the spread of AIDS.

Under the European Convention on Human Rights, interference with the privacy of an individual is acceptable only if it is "necessary in a democratic society for the protection of health". Sieghart says that because a positive AIDS test "will be perceived as an effective death warrant", and because mandatory HIV screening is not perceived by public health experts as an effective means of reducing the spread of infection, the severe adverse consequences of mandatory screening outweigh any benefits and such a scheme would not be justifiable under international human rights law.

Disclosure of the result of an HIV test to a third person without consent would be unjustifiable, says Sieghart, except if a patient refuses to take the necessary precautions not to pass on the infection to a known sexual partner, for example. Disclosure to another health worker is not justifiable on the grounds that he or she is at risk, says Sieghart, because health

workers should be trained in procedures needed to protect themselves.

Compulsory registration of suspected HIV carriers and the mandatory collection of identifiable personal information about AIDS sufferers or HIV carriers, without protection of that information by strict rules of confidentiality, would also be unjustifiable interference with privacy. But making it a criminal offence for an HIV carrier knowingly or recklessly to infect others might be justifiable on public health grounds.

Sieghart also says that estimating the prevalence of HIV infection by testing samples taken with the patients' consent, for other purposes, would not interfere with the right to privacy if the samples cannot be traced to the individuals from whom they were taken.

On no grounds would the prohibition of marriage for persons known to be HIV-positive or the compulsory testing for HIV of people who want to marry be justified. Neither would mandatory abortions or sterilizations for HIV-infected women, because this would be a violation of the human right to found a family.

The right to work is protected by international human rights law, so an applicant for a job could not be legitimately refused employment or be dismissed on the grounds that that person was HIV-positive, unless the absence of infection was a necessary occupational qualification or the infection would clearly and substantially affect job performance.

Christine McGourty

ANTARCTIC TREATY

Convention hits more trouble

Paris & Sydney

In a television interview last week, French Prime Minister Michel Rocard made a surprise declaration that France is not prepared to ratify a convention supplementing the Antarctic Treaty to limit mineral exploration in the Antarctic. "The convention improves the situation slightly", he said, but added that discussions should seek to go further.

The convention, which is the third major amendment to the 1959 Treaty, was initiated by New Zealand and was adopted in June last year. Of the seven nations that have territorial claims to Antarctica, France and Australia have still not ratified the convention.

The convention requires all mineral exploration projects to be submitted for international scrutiny and seeks to close a loophole through which signatory nations could carry out illicit operations through companies owned by non-signatories. Until the convention comes into force,

signatories further undertake to halt all mineral exploration. Although France has denied that it has plans to exploit Antarctic mineral wealth, Rocard's «non» will inevitably add grist to the mill of the environmental action group, Greenpeace. Over the past few years, Greenpeace has drawn attention to environmental damage allegedly being caused by the construction of a French landing-strip, accusing France of opening the way for mineral exploration under the guise of scientific research.

Australia last week put off its decision on the treaty. The minister for the environment, Senator Graham Richardson, wants Australia to sign, arguing that it is better to have an agreement that permits exploration rather than risk uncontrolled development. "Mineral developments, if they are to begin in any unplanned way, could lead to disaster", Senator Richardson said. With France's refusal it seems likely that negotiations will have to begin again in Wellington. Peter Coles & Tania Ewing

Post-mortem on failure

Moscow

FATE has dealt the Phobos project some cruel blows. Last September, due to an operator's error and its consequences, the first spacecraft was lost. So researchers' hopes were pinned on Phobos 2, the second of the two spacecraft aimed at orbits about Mars and its moon Phobos. But when the second probe approached the surface of Phobos, the onboard transmitter fell silent. Attempts to save the spacecraft were unsuccessful. So was it a complete failure or just a partial setback?

The project directors exposed themselves to criticism by their silence during the critical period. Information for the press dried up. No doubt, in the past, the whole affair would have been passed over in silence, but public pressure is now so strong that the engineers and scientists were eventually compelled to meet journalists and give an explanation.

Two opposing opinions stand out. Technical specialists believe that Phobos accomplished its main tasks, but scientists are convinced that the spacecraft had serious defects. This is a classic conflict between the manufacturer and the consumer, in this case between industry and science. Readers may be able to draw their own conclusions.

Vyacheslav Kovtunencko, the project's technical director, says that when it was decided in 1980 to embark on the comprehensive exploration of Venus, Mars, the Moon and the asteroids, the question arose whether to design a special space vehicle for each object or an all-purpose platform that could, with modification, explore them all. The second option offered benefits, and was approved.

Kovtunencko says that the Phobos project has shown that spacecraft built around a standard platform can perform complex tasks at the distance of Venus or Mars, bring soil samples from the Moon's far side and investigate the far side of the Sun. But he acknowledges that there were malfunctions and design defects, adding that these are inevitable in such complex projects.

Academician Roald Sagdeev, the project's scientific director, sharply disagrees. He noted that the successful Vega project (to Halley's comet) had been "up to the mark" and that, by carrying instruments from scientific organizations in nine countries, Vega had brought Soviet science "credit" and prestige.

Sagdeev said that the collaborators from 13 countries in the Phobos project had had to "overcome the resistance of financial bodies and, often, political resistance from opponents of the new thinking", particularly of the expansion of contacts with the Soviet Union. "We were sure that Soviet technology would give of its best",

but "it seems that *perestroika* has not reached that sphere".

Sagdeev also disagreed with Kovtunencko's "confident conclusion", saying that the essence of the project had been the exploration of Phobos. "The most original ideas and technological solutions were invested in this part of the programme, which has not been fulfilled."

Referring to his candidacy in the elections for the People's Chamber of Deputies, Sagdeev said that one of the points in his election programme was "the struggle for the restoration of the people's trust in space programmes". He hoped that the future Supreme Soviet of the USSR would uphold the interests of its electors in space research. "We'll have no future without space exploration", but "our style" must change.

Vladimir Pochukayev, head of the Phobos ballistics group, stressed the difficulty of the venture. The orbit of Phobos had been uncertain by hundreds of kilo-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Elusive moon — a Viking orbiter photomosaic of Phobos.

metres, but the spacecraft's flights had allowed it to be refined to within two or three kilometres. As well as photographing Phobos, the spacecraft had also studied the second martian moon, Deimos, the coordinates of which had been fixed with an accuracy of tens of kilometres. "I believe that ballistics experts have performed their task", Pochukayev said.

On the scientific programme, Professor Albert Galeyev, director of the Soviet Academy of Sciences' Institute of Space Research, said that the first stage of the project had been carried through successfully. X-ray images of the Sun had revealed solar flares whose intensity was exceeded only by the record outbursts of 1972. The magnetic field of Mars had been measured, as well as its radiation belts. But lack of time had meant that some studies had been unfinished.

On Mars itself, Professor Vasily Moroz explained that Phobos instruments had obtained data on minerals containing water of crystallization as well as on the atmosphere. A thermal chart of a part of

the martian surface had been made. But, he added, the Phobos spacecraft are "inferior in some respects to US interplanetary probes".

Professor Vladimir Lapygin, a specialist in control systems, made an organizational point. Noting that projects now "apparently need a scientific director", he complained: "we cannot say that someone is responsible for instruments, while others are responsible for the other and more complicated components".

Dr Arnold Selivanov said that the Phobos radio complex was an order of magnitude better than on any previous space probe, but that because it takes a signal 30 minutes to cover the distance between the Earth and Mars and back, it is impossible to control a probe remotely. The Phobos probe, he said, had insufficient independent compensating intelligence, yet even so it came close to success.

Selivanov praised the infrared scanning device mounted on Phobos 2 "at the very last minute", describing it as a thermal television set made by enthusiasts. Apart from thermal charts of Mars, the instrument had revealed the shadow of Phobos on the surface of the planet.

The full repercussions of the Phobos failure are not yet clear, according to Alexander Dunayev, chief of the Soviet Space Agency (Glavkosmos), who explained that future projects are now being re-examined. The fate of the Mars-94 project is not yet decided, while a decision on whether to carry out a regular cycle of studies of Mars in 1995 will apparently be taken in May.

The cost of the project is also much discussed. Yuri Koptev, a department chief of the Soviet Ministry of General Machine Building, says the overall cost was 272 million roubles, including the manufacture of Soviet instruments, the space probes themselves (Rb 51 million), the booster rockets, preparations for the launch and even the improvement of two powerful 70-metre radio telescopes in Yevpatoriya and Ussuriisk.

But Roald Sagdeev was quick to add to this "the equivalent of Rb 60 million in hard currency", what 150 enterprises abroad had spent on the manufacture of scientific instruments.

So what went wrong? Roald Kremnev, one of the project leaders, says that the last telemetry data showed the spacecraft to be spinning without proper altitude control. "Had one, two or three systems gone out of order?" One possibility is that the swarm of small objects about Phobos was responsible. Certainly some alien object appeared in the field of view of the stellar sensor towards the end. Was that a part torn away from the spacecraft or a piece of asteroidal material? Kremnev says that only continued analysis of the telemetry will make it possible to decide.

Mikhail Chernyshov, *Novosti*

Problems at Albert Einstein

SIR—We, the undersigned faculty members of the Albert Einstein College of Medicine, are responding to the article by Joseph Palca (*Nature* 338, 192; 1989). It is not our intention to comment upon the specific issues in this article. Our concern relates to the statement that "Einstein has had a poor track record in its attitude towards female faculty".

Having been associated with the Albert Einstein College of Medicine in New York for many years, some of us since its inception, we recognize that our opportunities and achievements in science, including the President's Medal of Science and membership of leading societies, such as the National Academy of Sciences, as well as of National Institutes of Health study sections and councils, derive to a large extent from our positive associations with Einstein. Since the college opened its doors in 1955, it has upheld its humane policy of equal opportunity for all, regardless of race, creed, political beliefs or sex. In fact, there is a high proportion of women among the Einstein faculty, including those in senior rank. Women did and do serve as departmental chairpersons. It would be difficult to find an institution with a better track record in its attitude towards female faculty than the Albert Einstein College of Medicine.

BARBARA K. BIRSHTEN (Professor of Cell Biology), OLGA O. BLUMENFELD (Professor of Biochemistry), SUSAN B. HORWITZ (Professor and Chair of Molecular Pharmacology), GERTIE F. MARX (Professor Emerita of Anesthesiology), ISABELLE RAPIN (Professor of Neurology and Pediatrics), BERTA V. SCHARER (Distinguished University Professor Emerita of Anatomy and Structural Biology), LADONNE H. SCHULMAN (Professor of Developmental Biology and Cancer), PAMELA M. STANLEY (Professor of Cell Biology), SALOME G. WAELSCH (Distinguished University Professor Emerita of Molecular Genetics), ORA M. ROSEN (Visiting Professor of Molecular Pharmacology, Former Professor and Chair of Molecular Pharmacology).

Albert Einstein College of Medicine,
Bronx, NY 10461, USA

SIR—The National Coalition would like to comment on the seemingly 'spontaneous' letter (above) signed by 10 women faculty of the Albert Einstein College of Medicine of the Yeshiva University supporting the college as non-discriminatory.

The letter flatly asserts that there is "a high proportion of women among the faculty at Einstein, including those in senior rank". It makes no statement about their salaries in comparison to male colleagues. We find the omission significant and glaring since, in the case of Sobel vs. Yeshiva University, a class

action case involving 40–60 women, the Court of Appeals found "blatant, systemic, sex-based wage discrimination". This case has been in court for 11 years and has cost the university more than \$2 million so far.

The assertion that women "do serve as departmental chairpersons" suffers from the same lack of comparison.

What perhaps is more indicative of the sincerity motivating this letter is the signature of Dr Olga O. Blumenfeld, who is on record in the faculty senate minutes as saying that the Sobel case accompanied much by drawing attention "to prior unequal treatment of Women Faculty at Einstein and in other medical schools and that Einstein has sought to correct salary inequities".

The letter's statement that "since 1955, (Einstein) has upheld its humane policy of equal opportunity for all," suggests that the signatories are attempting to placate an increasingly angry public, including several important women philanthropists, who are angry at the pattern of sexual discrimination at Einstein, an issue which has been raised anew by the Heidi Weissmann case.

The minutes of the faculty senate reveal outrage at the continuing waste of university resources and anger with the continuing pattern of discrimination. Dr Blumenfeld herself, reporting to the senate that Yeshiva was now taking the case to the Supreme Court, said with some exasperation, "I don't want to be involved in this case any more. . . I only want to express my own disappointment that the administration has never answered our resolution (urging settlement of the Sobel case) and never informed the senate why they are taking the actions they are taking". In another place in the minutes, Dr Blumenfeld says that ". . . the school, if it reached a settlement, would gain in reputation as a progressive institution".

The Coalition suggests that this supportive letter for Einstein has more to do with faculty concern about bad press than with the reality of discriminatory behaviour at their own institution.

LEONARD MINSKY

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in the Public Interest,
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SIR—I was given by Dean Purpura of the Albert Einstein School of Medicine a copy of a letter signed by 10 female faculty members at Einstein, written in response to an article by Joseph Palca about a plagiarism dispute between two faculty members at Albert Einstein School of Medicine. The signatories stated that Einstein gives equal opportunity to women, and has a good track record in its

attitude toward women.

I represent a philanthropic foundation that did *not* give an important contribution to this institution over three years ago because of the then 10-year-old Sobel vs. Yeshiva University case which was eating up funds that could be used for a better purpose. This case is still in litigation.

I find the letter signed by the ten female faculty members hard to understand. The faculty senate minutes of 24 June 1987, 10 February 1988, 9 March 1988 and 11 May 1988 paint a very different picture. Dr Olga Blumenfeld, who signed the letter to *Nature*, expresses in all of these verbatim transcribed minutes her intense frustration at the failure to settle this case, at the excessive university spending (close to \$2 million by 24 June 1987) and the lack of response on the part of the university to the faculty senate motions to negotiate a settlement.

Reflecting on Dr Blumenfeld's exasperation, I can only wonder why she signed the letter sent to you. As a philanthropist, I am further encouraged by this situation to investigate carefully all institutions that request funding, and to do my best to persuade other philanthropic organizations and individuals to demand that the institutions they support conform to the highest ethical and moral standards before they make such gifts and donations.

RITA J. KAPLAN

Rita J. and Stanley H. Kaplan
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New York, New York 10017, USA

No photocopying

SIR—There is a further point relevant to the discussion about the merits of photocopying research papers or sending for reprints. At the University of Manchester, an agreement between the university and the Copyright Licensing Agency forbids the photocopying of more than one article from a single issue of a journal. I do not know how many other institutions are subject to similar regulations, but the implication here is clear: if copies of more than one article from a single issue are to be obtained, it must be by sending for reprints.

D. L. MOSS

Department of Mathematics,
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Two birds. . .

SIR—Will it soon be possible to achieve superconductivity and fusion at the same temperature?

ROBERT HILL

Computing Service,
University of Leeds,
Leeds LS2 9JT, UK

Where next with peer-review?

Next week's conference at Chicago on the peer-review system as it applies to journals will rehearse old discontents about the system, but should also ask whether pressures on the system now can be sustained.

THERE seems to be a paradox at the heart of what is called the 'peer-review' process: researchers wishing to see their work in print prefer that it should appear in journals that send research articles they consider seriously to outside referees for appraisal and criticism, but are then frequently deeply offended if publication is refused in the light of what the referees may say.

But the seeming paradox is not a paradox at all. Rather, the umbrage caused by refused publication on these grounds may be likened to the resentment of the practice, in the old days, by which exclusive clubs would allow existing members to veto (or 'black-ball') new applicants for membership. To be accepted by one's peers is naturally preferable to acceptance by a group of people drawn at random and, conversely, to be rejected by them is doubly hurtful.

But there is more to peer-review than the apportionment of *amour propre*, as can be told from the programme for the meeting, billed as the International Congress on Peer Review in Biomedical Publication, which has been organized by the American Medical Association at Chicago next week. Perhaps inevitably, the peer-review process as such has become a subject of academic study. How consistent with each other are referees' opinions? Does the intervention of referees in the publication process bias the literature, perhaps in a conservative direction? How well do referees' opinions stand up to the passage of time? These are interesting questions, even if their relevance to the process of publication is not easily fathomed.

One difficulty is that the origins of the peer-review system are obscure. Half a century or so ago, most of the journals making up the then much smaller bulk of the scientific literature were published by scientific societies which, having appointed a board of editors consisting of people knowledgeable in the field, would trust them to decide what should be printed. Vestiges of that system persist, although the editorial boards of most journals have found it increasingly necessary to seek outside advice. Their collective expertise, however great, will not always allow sure judgements on questions such as whether a particular technique has been correctly carried out, or is an appropriate foundation for the inferences drawn from the data it provides.

General journals such as this, without a formal board of expert editors, naturally have an even more urgent need of outside advice, but usually have been slow to seek it. So much can be told by glancing at volumes of *Nature* dating from as recently as the 1950s. A little reflection, for example, will show that the brief letter in which J. D. Watson and F. H. C. Crick described what turned out to be the correct molecular structure of DNA (171, 737; 1953) could not have been through a particularly stringent review process.

There is internal evidence — the interval between submission (on 2 April) and publication (25 April) can hardly have left much time for careful scrutiny and criticism. The original article also includes statements that would not now survive in the hands of referees. Watson and Crick described a structure arrived at by model-building, adding "so far as we can tell, it is roughly compatible with the experimental data". Few referees would now let that pass, nor would they be happy with the much quoted "It has not escaped our notice that the pairing mechanism we have postulated immediately suggests a possible copying mechanism for the genetic material". "Mere speculation!" would be the complaint.

So why have those informal and, no doubt, genteel ways of deciding what to publish been forsaken? (All the contributions now appearing as *Articles* or *Letters* in *Nature* have been subjected to this kind of external criticism, but with the extra request of referees that they should also advise on the immediate interest of what is offered for publication.)

The circumstance that *Nature's* editorial staff are mostly not practising scientists is only part of the explanation. Even the gigantic increase in recent decades of the volume of material seeking an outlet in print, and the steady process by which sub-specialties become sub-sub-specialties, do not in themselves account for what has happened. More subtle changes in the nature of scientific publications are more telling.

First, and to the credit of the scientific community, there has been sustained and deliberate pressure to improve the standards of publications. When there is so much talk of how the literature is corrupted by false and even fraudulent reports, this beneficent tendency is too easily forgotten. People no longer wish to waste their time reading research reports

which include so little data that their conclusions cannot be verified. Nor do they rejoice when the authors of entirely respectable research go on to speculate about the general implications of what they have done (which is generally distrusted because it is a means by which the importance of a piece of research may be magnified artificially).

The other side of that coin, of course, is that research reports have become less palatable as literature. Most articles in most journals can now be read without difficulty only by those who happen to be working in a closely related field. But need the rule that rigour means difficulty of understanding be as closely followed as it is? In its admirable pursuit of meticulousness, the scientific community is in danger of overlooking the not necessarily incompatible and primary function of the literature, which is communication.

Another and more insidious pressure on the system is competitiveness — people's anxiety to be the first in print with reports of important or even unimportant discoveries. Everybody knows that the stakes are high. Publications, especially in journals known to be rigorous in their selection of what they publish, do determine promotion prospects and influence the likelihood of continuing research support.

But competition is in danger of getting out of hand. Journals such as this are now often presented with the uncomfortable need to decide whether loose use of language, or inadequate experimental data, are consequences of authors' haste or of some more sinister concealment of the whole truth. This does not happen often, but that it should happen at all is a serious matter, requiring the cultivation, by referees and all others concerned, of an over-suspicious frame of mind.

That, of course, is merely another reason why some means must be sought for abating the pressure to publish quickly, or to publish in leading journals, or even to publish at all. In the long run, there will have to be better means by which the academic community can make judgements of the quality of its members. One benefit would be that the literature would become more like literature, but nobody appears to have assessed how great would be the benefit if much of the time that referees now voluntarily lavish on other people's manuscripts could be used for better things.

John Maddox

A spot of luck for brown dwarfs

Ron Probst

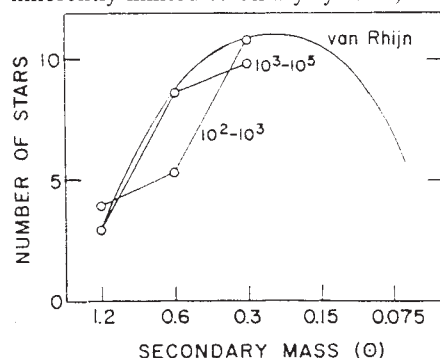
ON page 38 of this issue¹, D.W. Latham and co-workers report the discovery of an unseen companion to a nearby solar-type star HD114762. The companion has a mass most probably in the range of substellar brown dwarfs, and is in an orbit about its primary similar to that of Mercury in our own Solar System. The discovery was made serendipitously in the course of a long-term spectroscopic survey intended to establish the radial velocities of standard stars. Coming hard on the heels of similar but unsuccessful searches^{2,3}, it renews hope for other would-be discoverers of brown dwarfs and raises again questions about how, or even if, such binary systems relate to the problem of the unidentified dark matter in the disk of our Galaxy and the origin of planetary systems.

Since the work of Oort 30 years ago it has been known that there is a discrepancy between the mass in the disk of our Galaxy required to account for the kinematics of disk stars and that which is actually observed in the form of stars, gas and dust. The most recent work indicates that half the mass of the disk, though distributed like the visible material, is in some unseen form⁴. Very low-mass, faint stars and substellar objects known as brown dwarfs have been two favourite candidates for this dark mass. The increasingly complete census of the stellar population in the vicinity of the Sun rules out the former, in the opinion of many⁵. Renewed interest in the problem, the elimination of other candidates and the availability of new technical means have produced several systematic searches for brown dwarfs in recent years. There have been a few claimed discoveries and numerous null results. The discovery reported by Latham *et al.* seems to be the firmest yet.

Brown dwarfs are hypothesized to form in a 'starlike' way during the fragmentation and collapse of a molecular cloud, but within sufficient mass to ignite hydrogen in their interiors. Because the thermal energy reserve that arises from the gravitational collapse is limited, they quickly plunge to low surface temperatures and optical invisibility. Direct radiometric detection of a brown dwarf is best accomplished at infrared wavelengths (around 2 micrometres), but the lack of panoramic detectors has prevented a search for the large population of isolated objects which is necessary to resolve the disk mass discrepancy. Instead, direct searches have concentrated on seeking brown dwarfs that are companions to nearby known stars, either by looking for a flux excess in the integrated light or by imaging techniques. There have been some apparent successes^{6,7}. The difficulty lies in the

interpretation. The fundamental physical variable determining the classification of a brown dwarf is mass; however, the observables, luminosity and photospheric temperature, are strong functions of age, which is only loosely constrained for most potential target stars. Thus the precise nature, or even the brown-dwarf status, of such discoveries is ill-defined.

The technique employed by Latham *et al.* and others, searching for a periodic variation in the radial velocity of stars, is inherently limited to binary systems, but



Mass distribution functions for field stars (van Rhijn) and long-period-binary companions (periods in years). Does the companion of the short-period binary HD114762 fit in a similar distribution? (From ref. 8.)

the observable property is directly related to the fundamental one: the mass function is a straightforward combination of component masses and orbital parameters. Interesting limits can be put on companion masses and a valid frequency distribution of mass can, in principle, be derived with a sufficiently large sample. The discovery by Latham *et al.* was accidental. Previously, Marcy and Benitz⁷ had deliberately searched a somewhat larger sample of 70 low-mass stars with similar techniques and sensitivity. A few new stellar companions were identified, but no brown dwarfs. Campbell, Walker and Yang⁸ used a clever twist on conventional spectroscopic methods to survey 23 stars with much higher sensitivity, but again with a null result.

Why one such survey should be successful when others of similar extent or higher precision were not is an interesting question which it is premature to attempt to answer. The more fundamental question, however, is whether the population properties of brown dwarfs as binary companions tell us anything about a population of free-floating objects.

Here the waters become muddied. On the observational side, the fundamental contribution is that of Abt and Levy⁹, who combined a large-scale, systematic radial-velocity survey of solar-type stars with other data to derive the frequency of

occurrence of binaries of various kinds. They found that for systems with long periods (of decades or longer), the distribution function for companion masses has the same shape as that of field stars, down to masses a few tenths that of the Sun (see figure). By extension, one might argue that the frequent occurrence of brown dwarfs as companions in long-period systems would imply a large population of field objects. For the short-period systems — those amenable to radial-velocity searches — there is a strong observed preference for equal masses. This suggests either that the field-star mass distribution is strongly modulated by other effects in the formation of such systems, or that the formation process is basically different. Disentangling the original field star distribution (and, by extension, that of brown dwarfs) would be very difficult in the first case, impossible in the second.

On the theoretical side, considerable advances have been made in recent years in computational modelling of the star-formation process, with some attention being paid to reproducing binary star characteristics. Following the recent review by Boss¹⁰, there are two plausible formation processes. It seems that fission of a single, rapidly rotating protostar into two bodies — creating a short-period binary — may just work, but only for very-low-mass companions, such as that reported by Latham *et al.* Such systems would be special cases, with no relevance to any field-star population. The favoured process is the hierarchical fragmentation of molecular clouds into successively less massive subunits, which subsequently collapse to form stars. This can produce long-period binary systems in which the secondary mass distribution does mimic that of the field. However, for short-period systems, equal masses are preferred. In this instance the Latham *et al.* companion would be something of a freak, but a large enough well-defined sample might still imply something meaningful about the frequency of brown dwarfs in general. It is also possible that the theoretical treatment of short-period systems is incomplete, due to biases in the modelling process in favour of equal mass systems.

What about the formation of other planetary systems? Discussion of this has become *de rigueur* in papers on brown dwarfs, although the relationship is often tenuous and at least in part a matter of definition rather than physics. Work on star formation¹⁰ suggests that the lower mass limit for bodies which form by the collapse of a molecular cloud is 0.02 solar masses or more. The physics which determines this limit is unrelated to that which determines whether the collapsed object will ignite hydrogen, a circumstance which leads to the expectation of finding at least a few brown dwarfs among the stellar population. It is satisfying in this

regard that the companion to HD114762 is probably well within the brown dwarf mass range. Planetary formation is thought to follow the creation of a proto-planetary disk during the formation of solar-type stars. There is no indication that a massive body such as the companion to HD114762, either rocky or gaseous, would form in this way in such close proximity to a solar-type primary. The significance of the companion of HD114762 for planetary formation is then solely exclusionary — one more solar-type star which probably does not have planets. The results of Campbell *et al.*³ are more interesting in this regard. Although they found no brown dwarfs, their sensitivity to low-amplitude velocity variations was sufficient to penetrate the regime of planetary mass detection (about 1–10 Jupiter masses). They identified several candidate systems. Extensive follow-up observations will be needed adequately to test the reality of these systems.

For the present, it is exciting to see positive results in the search for brown dwarfs obtained from a relatively small sample. It is promising for the future that this

detection was achieved by straight-forward instrumental means on telescopes of moderate aperture. Uncertainties in the interpretation of results *vis-a-vis* the galactic-disk mass problem may be ameliorated by further observational work on the statistical properties of binary stars and by computational work with more generalized initial conditions. Those of us who search by other means can only encourage the radial-velocity observers, keeping in mind that well-defined and thorough programmes on carefully selected samples are the most likely to produce statistically meaningful results. □

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ECOLOGY

Communities oceans apart?

Stuart L. Pimm

MANY ecologists can drive to their study sites — land, lakes and the intertidal. They often choose to study the behaviour, population dynamics and interspecific interactions of organisms experimentally on plots of 1–10 square metres. They receive training in mathematics and statistics and read a distinct set of journals. Biological oceanographers, on the other hand, sample across vast tracts of ocean from boats, and document the physical and chemical effects on biomass for which their training prepares them. At a recent meeting*, terrestrial, aquatic, intertidal and oceanic ecologists considered the similarities and differences of their systems and methods. They generally failed to agree, and my list is not a consensus either. There was unanimity, however, that the different views are an essential ingredient in solving some critically important ecological problems.

The comparative approach is essential in ecology, where many ideas cannot be tested experimentally. Comparisons of the number of species in marine and terrestrial systems provide one example that illustrates the strengths and weaknesses of such broad contrasts. The total number of species on land may be much greater than in the oceans (J. McGowan, Scripps Institute of Oceanography). But

the numbers of species in poorly known tropical forests may only seem to be greater than in marine benthic communities because the latter are even less well known. Even if the difference is real, it could be explained because terrestrial communities are dominated by the excessively diverse insects, which are almost entirely absent from marine communities (H. Caswell, Woods Hole Oceanographic Institute). Do insects have particular features allowing this diversity, or does it result from the terrestrial environment? Suppose that the difference results from the environment. Terrestrial diversity increases with increasing environmental patchiness because patches of different sizes (gaps in a forest, say) have their own idiosyncratic species. Satellite imagery shows that the physical features of the ocean are very patchy (J. Steele, Woods Hole Oceanographic Institute). Yet the cold water rings in the turbulent flow of an oceanic current may contain species common to cold water rather than species unique to the patches. What does this say about the role of movement and dispersal in driving species richness? Dispersal processes on land and in the sea are likely to be very different.

Differences in population dynamics may be the least contentious comparison. Ocean food chains have at their base phytoplankton with very short lifetimes and large, top predators with long life-

times. On land, very long-lived trees and shrubs or slowly decomposing organic matter are at the base of the chain, with shorter-lived top predators and the shortest lived species sandwiched in the middle. (Of course, intertidal, benthic and coral-reef habitats are more like terrestrial habitats in this respect than the open oceans. A recurrent theme of the meeting was that the greatest differences are between planktonic and anchored communities.) Food-chain models show that the different patterns of longevity have a profound effect on community dynamics (R. Wiegert, University of Georgia). Given this difference in dynamics, it is perhaps surprising that many characteristics of food chains and food webs are so similar in terrestrial, aquatic and marine habitats (J. Cohen, Rockefeller University).

As significant as the differences in the systems studied is the difference in approaches and methods imposed by the different means of reaching study sites. No group is likely to export its ideas and methods to the other without the other seeing the potential profit in importing ideas (S. Levin, Cornell University). Nowhere is the importance of this more apparent than in considering the consequences of global climatic changes. The important questions of scale — for how long and how far physical variables are essentially unchanged — are commonplace in the marine literature yet are rarely addressed explicitly in the terrestrial literature (Levin). The characteristic spatial scales of physical variables in the ocean and atmosphere are the stuff of undergraduate lectures in oceanography, yet a review of these ideas (T. Powell, University of California, Davis) was news to many terrestrial ecologists. In contrast, an evolutionary perspective and details of behaviour, population dynamics and community interactions are probably better known on land. Yet what is critically important is how these biological details follow or resist the changes in physical variables, and this coupling has never been adequately studied.

Understanding how physical changes affect detailed biological interactions will come from hitherto unlikely alliances of ecologists. How should such alliances be encouraged? There are many places for ecologists to pursue field work, and there are summer courses galore to train students in practical skills. Yet there is no place for either researchers or students to think collectively or to analyse data. The pressing problems of the loss of biological diversity and environmental change suggest that this omission must soon be rectified. □

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* A Comparison of Marine and Terrestrial Ecosystems A workshop held in Santa Fe, New Mexico, 14–16 March 1989. Organized by John Steele.

Selecting buried residues

T. E. Creighton and Cyrus Chothia

ONE of the central problems of molecular biology, that of the relationship between the amino-acid sequence of a protein and the stability of its biologically active folded conformation, would be better tackled if more were known of the effects on stability of sequence alterations. Nature, which has been experimenting with protein stability throughout evolution, has provided many examples: the homologous proteins from different species whose different amino-acid sequences are compatible with the same function, invariably with essentially the same structure. Now, the insight into the basis of protein stability provided by those sequences is being greatly enlarged by protein engineering and site-directed mutagenesis. It is now feasible to make a protein with any amino-acid sequence and to study its structural and functional properties. The article by Lim and Sauer on page 31 of this issue¹ and that recently published by Bowie and Sauer² are interesting illustrations of what is possible.

Although the current fashion is to use site-directed mutagenesis to make specific mutations, there is much to be said for persuading nature to divulge the secrets of protein structure by means of a procedure selecting, as in classical genetics, for clones producing either active or inactive proteins³⁻⁶. The structural genes of the selected clones can then be rapidly sequenced to identify deleterious, neutral or beneficial mutations.

With the new recombinant DNA techniques of saturation mutagenesis, all mutations at designated sites can be made to occur. The telling question then becomes not only which mutations are found, but also which have failed the selection test. One limitation of the procedure is that the mutant proteins are selected *in vivo*. Although the variability of functional properties allowed by the selection procedure can be determined by examining the pertinent properties of a few appropriate mutant proteins under defined conditions, there is always a possibility that some unknown factor is of crucial importance *in vivo*.

Even so, Bowie and Sauer² have shown convincingly that the spectrum of amino acids tolerated at each position in a small protein is adequate to classify the general role of the residue at that site: whether it is involved directly in biological function, whether it is on the surface or is buried, and whether it is involved in regions of α -helices or β -sheets.

The main contribution to the free energy of protein folding comes from the hydrophobic interactions from burying non-polar residues in the interior. The

close packing of the interior determines how the α -helices and β -sheets are arranged to form the three dimensional structure⁷. For these reasons, attempts to determine the relationship between the amino-acid sequence and stability have been dominated by consideration of protein interiors.

In their paper in this issue, Lim and Sauer¹ concentrate on the seven residues that comprise the hydrophobic core of the known structure of the amino-terminal domain of lambda repressor. Previous analyses of protein structures, using comparisons of homologous proteins and the individual mutations tolerated in mutational studies, have demonstrated the limits in the volume and hydrophobicity of the amino-acid side chains comprising the core³⁻⁸. The new results confirm those results.

Lim and Sauer have also substantially extended that kind of analysis by performing mutagenesis simultaneously at three and four of the seven core residues. When multiple mutations are permitted, additional amino-acid replacements compatible with a folded functional protein are found at each position. Although they examined only three combinations of multiple mutagenesis, their results show that:

- The extent of the change allowed at a particular site is governed by the changes that occur at other sites.

- For a particular site, the mutations at the other sites may increase the range of

residues allowed, as at residues 18, 36, 40 and 47, or restrict the range, as at residues 57 and 65.

- The volume variations that can be accommodated by a functional protein increase with the number of mutations.

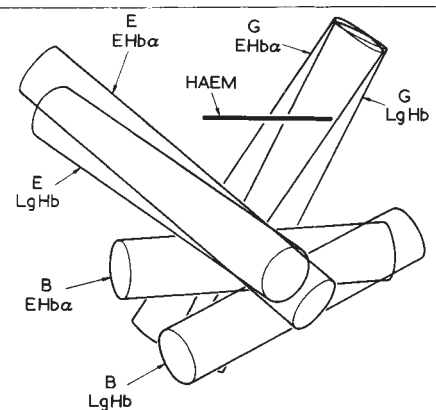
Mutated sites	Allowable volume ($\text{\AA}^3$)
1	531-567
2	526-585
3	500-585

- Not only is the volume of the core important, but the side chains must also be able to pack together with adequate density. An extra methyl group may be acceptable in one position but not in another.

These experiments clearly show that the amino-acid side-chains permitted at each position are determined by those present at neighbouring positions. The results have most interesting implications for the molecular mechanisms involved in the evolution of protein structures.

The sequences and structures of homologous proteins show^{8,9} that changes in the volume of buried residues in related molecules are not complementary, but are accommodated by changes in structure. For distantly related proteins, the major α -helices and β -sheets are shifted relative to each other by several angstroms, and the peripheral loops and secondary structures have different folds. The limitations on sequence changes arise from the need that a close-packed hydrophobic core should stabilize the structure, from the structural requirements of the site involved in catalysis and recognition (see figure), from the sequence changes taking place essentially one at a time and because

Mutations and coupled structural changes^{8,9}. For two distantly related globins, the position of the B, E and G helices is shown relative to the haem. The planar haem is represented by a thick line and the α -helices are represented by cylinders. The back of the haem pocket is formed by the G helix and the bottom by the E helix. The G and E helices are not in direct contact but pack on opposite sides of another helix, B. This figure shows the positions of these helices in the α -subunit of horse haemoglobin (EHb) and in lupin leghaemoglobin (LgHb). Only 19% of amino-acid residues of these two globins are the same and those that form the buried interfaces between the helices are very different. In the lupin leghaemoglobin, the volume of the side chains that form the interface between the B and E helices is 45% greater than in horse haemoglobin, and the volume of the side chains at the interface between B and G is 15% smaller⁹. The differences in the volumes and shapes of the interface residues change the geometry of the helix packings. Relative to the B helix, the positions of the E helices in the two structures differ by a shift of 7 $\text{\AA}$ and a rotation of 45° and the G helices by a shift of 3 $\text{\AA}$ and a rotation of 35°. Although relatively large, these movements are coupled so that the positions of the residues in the E and G helix that form the haem pocket have only small differences in position, about 1 $\text{\AA}$. Similar, though smaller, coupled changes have taken place at the other helix interfaces. Thus changes in structure allow the wide divergence of sequence. The structural change introduced by a single mutation will be small, but the cumulative effect of multiple changes must be consistent with function. Thus the viability of a mutation is determined in part by the structural changes produced by previous mutations and will, in its turn, influence later sequence changes.



the individual changes must not be too costly in free energy. This view of protein evolution is strongly confirmed, and extended, by the experimental results now reported^{1,2}.

Current theories for protein stability are largely empirical and semi-quantitative. The experimental data on the relationship between protein stability and amino-acid sequence now accumulating provide a great opportunity for a thorough theoretical analysis. The types of limited changes in amino-acid sequence being produced by mutational studies are ideal for testing detailed models of protein stability, so it is incumbent on theoreticians to develop them. At the same time, experimentalists must design tests and interpret their results in the light of the

theoretical ideas that do exist. It is time for a constructive dialogue between the two camps. □

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NUCLEOSYNTHESIS

Signals from the Big Bang

Craig J. Hogan

ATOMIC nuclei are manufactured and destroyed in a wide variety of astrophysical environments. The abundances of the various nuclei carry important information about those environments throughout the history of the Universe, including of course the early Big Bang itself. But because so much has happened since the Big Bang during the evolution of our Galaxy, it is a knotty problem to untangle the evolutionary history of the elements back to the beginning. At a recent meeting*, where astrophysicists re-examined the available evidence, many felt that the standard Big Bang theory could benefit from some modifications.

The standard view for more than 20 years has been that helium and deuterium originate in the Big Bang (although the former is somewhat enhanced and the latter partially destroyed by galactic events). This basic idea is alive and well, and is still being refined. Current observations of helium abundances in metal-poor nebulae produce numbers accurate to the third decimal place (B. Pagel, Royal Greenwich Observatory); the last digit is, however, subject to uncertainty about stellar production. The standard Big Bang model, which assumes that the primordial Universe was homogeneous, produces the correct abundances for these elements only if the density of ordinary baryonic matter is less than about a tenth of the value needed for a 'flat' Universe (a Universe with enough matter just to halt its eternal expansion) (G. Steigman, Ohio Univ.). It also prohibits there being more than four species of light neutrino, in agreement with recent accelerator experiments constraining the number to be less

than five (D. Cline, Univ. California, Los Angeles; D. Schramm, Univ. Chicago). Theoretical uncertainties in Big Bang predictions are being reduced through improved understanding of the relevant weak-interaction parameters (S. Freedman, Argonne National Lab.).

Many talks were devoted to testing a modification of the standard view which J. Applegate (Columbia Univ.) and I put forward several years ago. This is based on the idea that small-scale inhomogeneities developed in an early first-order phase transition during the Big Bang (the mechanism that is most often cited being the quantum-chromodynamic transition effects introduced by Witten). We suggested that these could have led to radical departures from the standard model by allowing some regions to be very neutron rich at the time of primordial nucleosynthesis. The most vocal advocate of this idea, W. Fowler (California Institute of Technology), whimsically but unashamedly referring to the standard model (which he helped to invent) as the "obsolete Big Bang", emphasized the attractiveness of having a nearly flat Universe containing only baryons (nuclear matter) and no 'exotic' matter.

Current models of this kind are being rapidly improved by the inclusion of additional effects, but remain idealizations of an inherently complex situation. Their predictions are therefore less ironclad than those of the standard model, although they adhere to a generic pattern: they often produce less helium and more deuterium, which is what allows a much higher baryon density in the Universe, but they also tend to give a higher lithium abundance and possibly a primordial enrichment of very heavy elements that are traditionally thought to be synthesized

in stars only. This last effect can occur even if the neutron enrichment is rare enough to leave the standard light-element abundances intact, whatever the mean baryon density, so it can be regarded as a direct signature of a first-order phase transition. Two important themes of the meeting were therefore to discover what the primordial lithium abundance actually is, and to understand heavy-element abundances in very metal-poor stars.

Lithium is seen in old and young stars, and in the interstellar medium. The general pattern that is observed (L. Hobbs, Univ. Chicago; A. Boesgaard, Univ. Hawaii) is that lithium is fairly abundant in gas and young stars (of the order of 10^{-9} by mass) but that old stars have a lower abundance by perhaps a power of ten. The question is, which, if either, of these abundances is primordial? Either the initial abundance was low, requiring that lithium was produced suddenly and with remarkable uniformity in the early Galaxy to generate its present high level, or the initial abundance was high, requiring substantial and remarkably uniform depletion via the synthesis of heavier elements in old stars to give its present low level.

Boesgaard argued on empirical grounds that the latter process seems indeed to be occurring in old population I stars, so that maybe we should also expect it to have happened in the older population II. We learned of recent theoretical models of S. Vauclair which seem to explain this uniform lithium destruction through convection and burning. But L. Krauss and C. Deliyannis (Yale Univ.) argued persuasively that their own stellar-evolution models could not explain such a small scatter in the lithium if it has been strongly affected by depletion. Some mechanisms were put forward (D. Dearborn, Livermore) by which early lithium enrichment could indeed occur. So in spite of considerable effort we still do not know where to look for primordial lithium, although we can now address the problem at a much more sophisticated level.

Considerable progress is being made in discovering the distinctive metal enrichment patterns in metal-poor (population II) stars, which are different from those of population I in many respects (R. Peterson, Harvard-Smithsonian Center for Astrophysics). Systematic searches for very metal-poor stars (T. Beers, Michigan State Univ.) promise to deliver a large, well-controlled statistical sample of the first stars to form, which should advance our understanding of early galactic chemical evolution and possibly allow us to discover a primordial component of heavy-element enrichment at the levels predicted by alternative cosmologies. □

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* Aspen winter conference *Observational Constraints on Primordial and Early Galactic Nucleosynthesis*, Aspen, Colorado, 15–21 January 1989.

Evolution in hidden forests

Christopher J. Cleal

PALAEONTOLOGICAL evidence now suggests that flowering plants (angiosperms) may have evolved first in the middle Mesozoic, possibly the late Jurassic, then radiated into different groups in the late Cretaceous. There have, however, remained lingering doubts about the quality of the early angiosperm fossil record. Do the fossils really reflect the early evolution of angiosperms as a whole, or are they just the remains of a group of species which happened to favour lowland habitats? To try to resolve this problem, Martin, Gierl and Saedler have applied the 'molecular clock' principle to nine extant angiosperms, representing six subclasses of both dicotyledons and monocotyledons. These authors report their rather startling results on page 46 of this issue¹.

The pattern of divergence of the lineages represented by these nine species is perhaps not surprising. The oldest divergence is of the monocot and dicot lines, followed later by the concurrent divergence of the five dicot subclasses. The primary division of the angiosperms into monocots and dicots is widely recognized, and the early divergence into these two lineages lends support to this. But the timing of these divergences is more problematic: the monocot–dicot divergence is thought to have occurred in the Carboniferous (319 ± 35 million years ago), and the dicot subclass divergence in the Permian (276 ± 33 million years ago). But this is about 150 million years before the earliest indisputable angiosperms appear in the fossil record.

Martin, Gierl and Saedler point out that, at face value, these results suggest that the angiosperms have a long pre-Cretaceous history, as has been argued by Axelrod². If this is true, there must have been an angiosperm homeland — a hypothetical 'upland' area where the group first appeared, perhaps in the Triassic or Permian, but where it would not be preserved in the fossil record. Angiosperms are supposed to have evolved in these hidden forests for a considerable time, until the Cretaceous, when they developed characters that made them pre-adapted to lowland habitats. Only then were they able to make their appearance in the fossil record. This view has recently become unpopular, mainly through the work of Hickey and Doyle³ and Doyle⁴, who have demonstrated an apparently plausible phylogeny for early angiosperms in the Cretaceous fossil record. But it is surely something of a coincidence that, of all the terrestrial habitats that were available, the flowering plants just happened to appear first in lowland, sediment-accumulating areas.

I suspect that the truth lies somewhere in between: angiosperms probably did evolve in so-called 'upland' areas, where fossils were unlikely to be preserved⁵, but not as long ago as the Triassic or Permian. It seems unlikely that the flowering plants, which are today so environmentally adaptable, took 100 million years to occupy the lowlands. There are more problems if the first appearance of the angiosperms was in the Carboniferous; not only is there an extra 50 million years or so, during which angiosperms were unable to descend to the lowlands, to account for, but where were the angiosperm ancestors? They are widely believed to have been among the Mesozoic seed-plants, such as the bennettites and corystosperms⁶, but there is no unequivocal evidence that these groups existed before the Triassic. Is it necessary again to postulate a long 'upland' history for these groups? Perhaps Long's hypothesis⁷ needs to be re-examined. Long suggested that angiosperm ancestors would be found among Lower Carboniferous pteridosperms, but subsequent cladistic analyses do not support this idea^{8,9}.

Martin *et al.* alternatively suggest that their results may reflect a polyphyletic origin for the angiosperms. The postulated dates of divergence are those of the most recent common ancestor and, if angiospermy arose along several distinct

ANGIOGENESIS

Successful growth of tumours

Russell Ross

THE successful growth of tumours is dependent on the process of vascularization (angiogenesis), but it is not known how these processes are related to each other. On page 58 of this issue¹, however, Folkman *et al.* clearly confirm that there is a correlation between the presence of factors that stimulate tumour growth and angiogenesis. Furthermore, Rastinejad *et al.*² have discovered a previously unknown inhibitor of angiogenesis which seems to be produced by cells when they are capable of expressing an active cancer-suppressor gene. The loss of this inhibitor activity occurs concomitantly with expression of both angiogenesis and tumorigenesis².

In their studies reported in this issue, Folkman *et al.*¹ examined genetically engineered mice expressing an oncogene in the beta cells of the pancreatic islets. As a consequence, these cells demonstrate a heritable capacity to proceed through the steps of tumorigenesis — from normal to

lineages, this common ancestor need not itself be an angiosperm. Several authors have argued for angiosperm polyphyly^{9,10}, but the presence of unique characters in the group, including double fertilization, provides a strong argument in favour of monophyly^{8,9}. It is difficult (but not impossible) to see how these characters all appeared independently in several lineages.

Martin *et al.* provide a thought-provoking study, but the marked discrepancies between their results and the fossil record make it essential that their method is robust. As a geologist, I am unqualified to enter into such a debate, but, if I understand the argument of Martin *et al.* correctly, the dating of the divergences depends on a constant rate of change in nucleotide sequences over long periods of time. I wonder about the validity of this argument, and even how it could ever be tested, other than by comparisons with the fossil record? The molecular clock was clearly ticking in the angiosperms, but was it keeping the right time? □

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hyperplastic to neoplastic. Folkman *et al.* find that in some isolated hyperplastic islets *in vitro*, angiogenic activity appears before the neoplastic transformation, suggesting that these hyperplastic islets induce angiogenesis by secretion of angiogenic molecules. Similar activity would presumably occur when hyperplastic islets become vascularized *in vivo* at a frequency correlated with incidence of tumour formation.

Folkman *et al.* interpret their results to imply that the angiogenic capability expressed by a given islet cell develops because of changes in the hyperplastic islets during the pre-neoplastic period, presumably after they have become committed to progressing to neoplastically transformed cells. In particular, Folkman *et al.* say that their data indicate a correlation between angiogenesis and the start of vascularization that precedes tumour formation (or that may be partly responsible for the transition from hyperplasia to

neoplasia). They state that "this abnormal cell proliferation then sets the stage for additional genetic or epigenetic secondary events. Induction of angiogenesis appears to be one of these events that is important for the conversion of normal epithelium into a cancer."

What Folkman *et al.* are suggesting should not be confused with the tumour progression and the heritable changes that take place in the DNA of individual cells that have undergone transformation. Perhaps the most important consequence of cellular transformation that allows a tumour to survive as a mass of transformed cells may be the ability of the tumour cells to stimulate their own vascularity by the induction of angiogenesis.

Although these observations¹ demonstrate that proliferating cells express the oncogene in the angiogenic hyperplastic islets, there are other explanations to consider in the evaluation of these interesting and potentially important observations. For example, several normal cellular genes, which are either repressed or not expressed when the cells are not transformed, often become expressed after the cells undergo transformation. This could result from lack of suppression of the normal cellular gene by the transformed cell, or through signals that somehow once again turn on a function that may have existed during embryogenesis or at some other time in the life of the cell, but which become repressed during adulthood.

It has been observed in several laboratories that neoplastically transformed cells express the normal cellular genes for molecules such as platelet-derived growth factor², fibroblast growth factor³ and transforming growth factor- α ⁵ (all of which may be angiogenic), whereas under most circumstances these normal cellular genes are not expressed by the adult cells. Platelets are a source for angiogenic molecules, including platelet-derived growth factor⁶, and an angiogenic platelet-derived endothelial-cell growth factor was recently discovered, purified, cloned and expressed by Heldin and colleagues⁷. Most other normal cells do not secrete angiogenic molecules except under pathological conditions or under conditions associated with embryogenesis, growth, development or wound repair. Thus it is possible that although angiogenesis is necessary for successful tumour growth and development, angiogenesis *per se* may not necessarily be related to the onset of oncogenesis at the cellular level.

Because growth-factor gene expression is a manifestation of oncogenic change, and this is particularly true for several angiogenic growth factors, is it possible that expression of genes encoding these growth factors might be useful as early markers of the transition from a hyperplastic to a pre-neoplastic or frankly neoplastic state? If this is the case, which suppressor genes or growth-factor genes should be looked for? The need is for cellular markers that delimit the change from a hyperplastic state, which is presumably reversible, to a neoplastic state, which is presumably irreversible with conventional treatments.

NONLINEAR DYNAMICS

Shocking optical solitons

Peter L. Christiansen

ALTHOUGH soliton, or solitary wave, phenomena arise in many areas of physics (see my previous News and Views article¹ for example), the single-mode optical fibre is especially convenient for their experimental study. Owing to the nonlinear interaction between light and medium in these fibres — the refractive index varies with light intensity — the normal tendency of light pulses to disperse can be negated. A recent twist to this story was added last year when Weiner *et al.*² showed that dark pulses, short breaks in an otherwise continuous laser beam, could also be made to propagate stably. And in new work³, Rothenberg and Grischkowsky observe optical 'wave breaking' attributable to interference effects at the edges of sharp optical solitons.

Soliton effects, like manifestations of chaos, are closely studied aspects of nonlinear dynamics. Interest has grown as increased computing power has made it possible to integrate the evolution of real dynamical systems, which are always more complex than their classical linearized

sequent alterations in the cell's DNA. Clearly, angiogenesis is critical to the process of tumorigenesis and is undoubtedly important, although alone not sufficient, for successful tumour formation. Understanding the controls for each of these steps, their relation to one another and the role of appropriate suppressor molecules that individually control angiogenesis as opposed to oncogenesis will shed further light on this process.

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simplifications. For optical solitons there are practical as well as theoretical implications. Optical pulses that retain their form over long distances have obvious applications in communications technology.

In 1973, Hasegawa and Tappert⁴ proposed that self-phase modulation, attributable to the intensity-dependent refractive index of some materials, could be used to compensate for group-velocity dispersion (a consequence of the frequency dependence of the refractive index). As a result, optical solitons can propagate without distortion. Since then, soliton propagation of bright optical pulses has been verified in experiments⁵ performed in regions of the spectrum for which the fibre has a negative dispersion (wavelength $\lambda = 1.3 \mu\text{m}$ in standard single-mode fibres). The most dramatic demonstration, the transmission of 55-picosecond (ps) pulses through 4,000 km of fibre, was reported by Mollenauer and Smith⁶ last year. The authors had to use the nonlinear methods just described to stabilize the pulse against spreading, along with

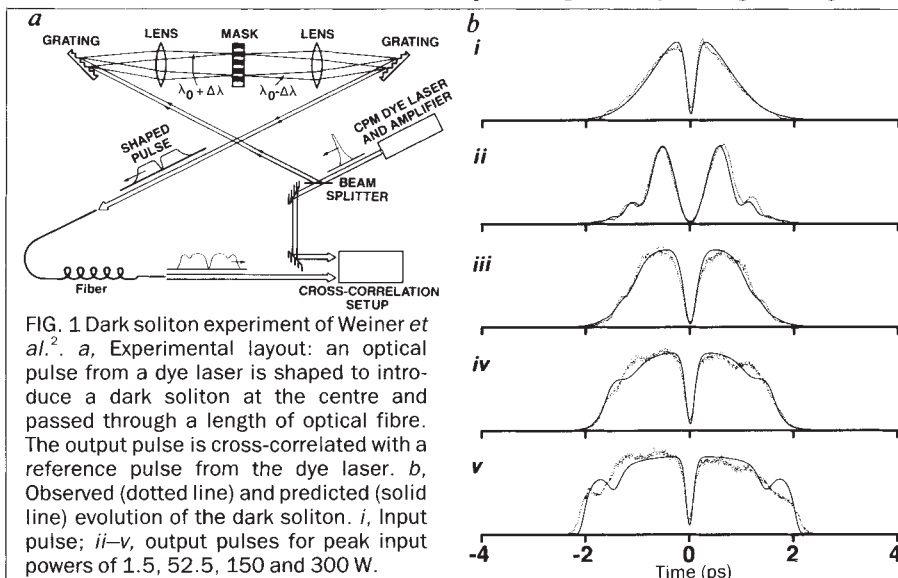


FIG. 1 Dark soliton experiment of Weiner *et al.*². *a*, Experimental layout: an optical pulse from a dye laser is shaped to introduce a dark soliton at the centre and passed through a length of optical fibre. The output pulse is cross-correlated with a reference pulse from the dye laser. *b*, Observed (dotted line) and predicted (solid line) evolution of the dark soliton. *i*, Input pulse; *ii-v*, output pulses for peak input powers of 1.5, 52.5, 150 and 300 W.

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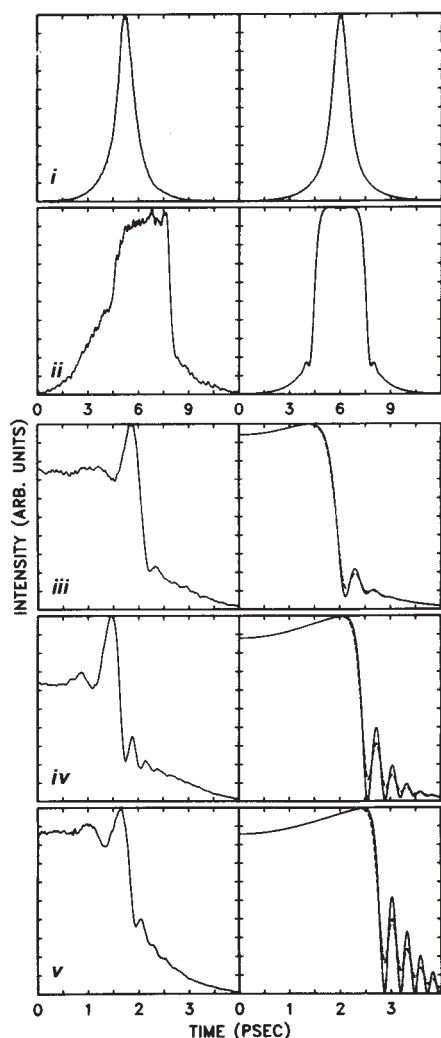


FIG. 2 Optical shock front formation and wave breaking as observed (first column) and predicted (second) by Rothenberg and Grischkowsky³. *i*, Input pulse; *ii*–*v*, output pulses for peak input pulse powers of 125, 250, 500 and 750 W. With increasing power (which accelerates the process), the formation of the shock (*ii*, *iii*) and wave breaking indicated by the appearance of 330-fs oscillations (*iv*, *v*) are clearly visible.

Raman amplification to overcome absorption and scattering losses.

For positive dispersion ($\lambda < 1.3 \mu\text{m}$), in the visible region, bright pulses cannot propagate as solitons, and the interaction of the nonlinear index with the group velocity dispersion leads to spectral and temporal broadening of the propagating pulses. For both signs of dispersion, the experimental results are in quantitative agreement with the predictions of the nonlinear Schrödinger equation, of fundamental importance because this equation represents the general situation of dispersive wave propagation with weak nonlinearity.

However, for positive dispersion the nonlinear Schrödinger equation admits a 'dark-soliton' solution⁷, which is a localized intensity dip in a constant intensity background. Mathematically this is an antisymmetric function of time with an abrupt phase shift of π and zero intensity at

its centre. Other dark solitons with a reduced contrast and a lesser, more gradual phase modulation also exist. Recently, Weiner *et al.*² generated 100–200-fs dark antisymmetric pulses on background pulses of red light, 1–4 ps in duration. These were passed through a single-mode optical fibre 1.4 m long, five times the characteristic length for soliton propagation of these pulses (Fig. 1*a*). The pulses have to be tailored to the right form before they can propagate as solitons: in this case, the fundamental wave form was obtained with spatial masking in a temporally non-dispersive lens and grating apparatus. Both the broadening of the dark pulse at lower powers and the narrowing of high powers (Fig. 1*b*) are predicted in the numerical simulations.

Also predicted in the nonlinear Schrödinger equation are so-called 'wave breaking' effects of solitons^{7,9}. High-resolution experiments by Rothenberg and Grischkowsky³ confirm this. In water waves, wave breaking occurs because the group velocity of the wave is greater at the peak than at the edges: the wave overtakes itself. Similarly, in nonlinear optical fibres, the effects of self phase modulation and positive group-velocity dispersion combine to make the pulse more rectangular (reshaping) and to impose a range of frequencies through the pulse (chirping). The edges of the rectangular pulse overtake the low-intensity wings: because of the differing frequencies in the pulse and in the carrier wave, interference causes intensity oscillations to appear at the leading edge — optical wave breaking (Fig. 2). Rothenberg and Grischkowsky observe this optical intensity-shock formation and subsequent wave breaking at various stages by varying the intensity of the input pulse and probing the output pulse with a resolution of 120 fs.

These elegant experiments do not merely verify numerical predictions of purely theoretical interest. Optical communications systems are currently based on linear techniques. It is an intriguing possibility that stable optical pulses could be used to increase the density of information in data transfer. This possibility has been substantiated by the 4,000 km transmission demonstrated in the laboratory by Mollenauer and Smith⁶. □

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Paradise delayed

OVER the past century or so, the age of adolescence in Western society has dropped by three to five years. Daedalus points out that this trend is quite wrong. In primitive society, a child entered adolescence with all the knowledge needed to become an economically useful adult and an effective parent. By contrast, children in our increasingly complex society need an ever-longer childhood to acquire the knowledge necessary for citizenship. But ever earlier in this process, puberty intrudes. Teachable children suddenly become rebellious and intractable adolescents, rightly resentful of the many further years of education they must face before becoming functionally adult citizens. Between the natural child and adult stages we have created an artificial teenage one.

So Daedalus is looking for ways to retard adolescence. It seems to take off when the child reaches a crucial weight; thus it is later in poorly fed societies where it takes longer to reach this threshold. It is also later at high altitudes — Daedalus reckons that the lower *g* at those heights reduces the body's perception of its threshold weight. On this argument, space-borne children should never reach adolescence at all. But this is hardly a practical solution to the problem.

The most appealing approach would be to 'reset' upwards the crucial weight at which adolescence is triggered. This can happen quite naturally: apparently normal children sometimes reach puberty extremely late. Nobody quite understands the hormonal mechanism; DREADCO's endocrinologists are comparing such children with their fellows, looking for some hormone or metabolite in the bloodstream whose concentration rises with age and weight, but is lower for the delayed adolescents. Once this crucial puberty-trigger has been identified, it should be possible to counter it. A suitable silicone implant could leak a chemical antagonist, DREADCO's Delay[®] into the bloodstream, holding off puberty to a more socially convenient age.

Upwardly-mobile, aspiring parents should queue to have their children Delayed. Their eager, untroubled offspring will sail through the whole educational system without being sidetracked by the angst and friction of adolescence. Maturing controllably late, they will enter physical and mental adulthood together, dodging the feckless, useless teenage period with its introverted sub-culture. By the time they crave children of their own, they will be economically fit to have them. Their late development could be wonderfully creative. Many scientists and mathematicians do their best work very early, before the clear and penetrating gaze of youth is distracted by the escalating problems of maturity.

David Jones

Ozone and the greenhouse effect

SIR—Two global air-pollution problems — ozone depletion and the greenhouse effect — are linked in many ways. Most of the so-called source gases from which ozone-destroying radicals are produced in the stratosphere, or which otherwise intervene with the ozone photochemistry, contribute to the greenhouse effect. Indeed the anthropogenic changes in the ozone layer (particularly the altered vertical distribution) also add to the greenhouse effect. Conversely the greenhouse effect, particularly its CO₂ component, produces stratospheric cooling, which reduces the effect of CFCs in causing ozone depletion in the upper and middle stratosphere. Thus the feedback between ozone depletion and global warming is partly positive and partly negative.

The increase in the CO₂ content of the atmosphere (the biggest single contributor to the greenhouse effect) results from the still steadily rising use of fossil fuels. But not all the emitted CO₂ stays in the air; there is an almost equal partitioning between atmosphere and ocean which is governed by an equilibrium between the partial pressure of CO₂ in the atmosphere and in the surface water (determined by the temperature-dependent solubility of the gas). The partial pressure of CO₂ in sea water is linked to the total CO₂ (CO₂ + HCO₃⁻ + CO₃²⁻). Because solubility increases with decreasing temperature, the tropical ocean is a source, and high-latitude oceans a sink, for atmospheric CO₂.

We must also consider biological processes. Phytoplankton assimilates CO₂ and can thereby lower its partial pressure in the surface water, leading to increased uptake of the gas from the atmosphere or reduced emission into the air. Dead organic matter or carbonate shells produced by organisms settle through the water column and transfer total CO₂ (as well as nutrients) to the deep sea.

Recent investigations show that increased ultraviolet-B (UVB) radiation (a consequence of a total ozone decrease) would hamper the photosynthesis of phytoplankton¹ in the ocean's surface layer, resulting in an increase of CO₂ partial pressure; the parallel rise in atmospheric CO₂ content leads to an enhancement of the greenhouse effect.

As our knowledge of the influence of increased UVB on phytoplankton photosynthesis is far from adequate, considerable work in this field is needed. There is the possibility of an adjustment by pigmentation and different plankton types may respond differently.

The continuous mixing of the ocean surface layer makes it difficult to calculate the reduction of photosynthesis that would result from an increase in UVB and

the resulting change of CO₂ partial pressure in the exchange layer. The exposure of a given phytoplankton unit changes rapidly, which may reduce the influence of UVB because the penetration depth of the visible light needed for photosynthesis is considerably larger than that of UVB. Experiments with increased UVB in a watertank may therefore produce misleading results; but it is difficult to carry out such experiments in the open ocean.

Another difficulty in assessing quantitatively the link between changes in the ozone layer and the CO₂ contribution to the greenhouse effect lies in the geographical diversity of the ocean surface conditions that are relevant to the ocean-atmosphere exchange processes. The high-latitude uptake and the low-latitude emission regions may be affected very differently by changes in the ozone layer.

It seems that the Antarctic (circumpolar) ocean plays an important role in this problem, because there the nutrients necessary for plankton growth (NO₃⁻, PO₄³⁻) are abundant due to strong vertical mixing with the deep sea. Although the Antarctic ozone hole is at present more or less confined to the continent, the related ozone loss around 60° S was already of the order of 10 per cent during the past 10 years². It can therefore be assumed that UVB has already increased considerably in this region, which, subject to the uncertainties mentioned above, could lead to a significant reduction in CO₂ uptake from the atmosphere in the Southern Hemisphere.

Changes in photosynthesis and thus in phytoplankton growth may also influence the atmosphere by reducing the emission by plankton of certain chemicals, in particular dimethylsulphide which may affect cloud formation through the production of sulphate condensation nuclei by oxidation in the atmosphere³. The consequent reduction of the Earth's albedo would contribute further to an increase in atmospheric temperature. The uncertainties associated with this effect are, however, considerable.

Further information on three factors — inhibition of photosynthesis in phytoplankton by increased UVB levels in the open ocean, the influence of such a change on the CO₂ exchange with the atmosphere, and the geographical distribution of such changes — should allow us to estimate the influence of ozone depletion on the atmospheric CO₂ content and thus on the greenhouse effect. The problem clearly calls for close cooperation between

biologists, oceanographers, atmospheric physicists and chemists.

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Pulsar evolution

SIR—Gravitational radiation may be controlling the evolution of the pulsar in supernova 1987A. If the reported 1968.63-Hz frequency¹ of the optical pulsations is the rotational frequency F of the neutron star², the rate of change of F resulting from emission of gravitational radiation driven by the CFS instability³⁻⁵ may exceed, by a factor of $\geq 10^4$, the rate resulting from electromagnetic and accretion torques. Observational determination of the period derivatives could provide the value of the viscous-damping timescale τ_v . The frequency f_m of the most unstable mode may also be observable.

It is reasonable to assume that this neutron star was born with the maximum rotational frequency allowed. The possible presence of a companion in an 8-hour circular orbit of comparable angular momentum¹ would imply that the collapsing core of the supernova had too much angular momentum for the neutron star to accept. The sharpness¹ of the pulsation frequency indicates that viscous processes brought the star into uniform rotation. A limit can be placed¹ on the present evolution timescale of this frequency: $T_0 = |F/(dF/dt)| > 600$ years.

Relativistic neutron stars rotating close to their maximum rate $F(\text{max})$ are unstable to the growth of sinusoidal ($\exp(im\phi)$) modes driven by gravitational radiation³⁻⁶. All modes whose growth time τ_m is less than τ_v (typically $m = 3-5$) will be excited⁷. Their inertial frequency lies in the range $400 \text{ Hz} < f_m < F$ and might also be detectable in the electromagnetic power spectrum⁸. Gravitational radiation at this frequency has an amplitude $h < 10^{-23}$, which is below current thresholds for detection⁸. This mode frequency is related to the rotational frequency by $f_m \approx m(F - F_m)$, where F_m , a decreasing function of m , is the rotational frequency required to excite mode m in the absence of viscosity. The loss of angular momentum through gravitational radiation generated by the dominant mode m leads to the slow-down rate

$$(dF/dt)/F_m = -(C_m/\tau_m)[M(\Delta R)^2/I] \quad (1)$$

Here C_m is an approximate constant of order unity, the growth rate $1/\tau_m = (1/\tau_v)(F/F_m - 1)^{2m+1}$, and the mode amplitude ΔR is proportional to $\exp[(t/\tau_m)]$.

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— (t/τ_v)). The constant τ'_m (proportional to $(GM/Rc^2)^{-(m+1/2)}$) (refs 9,10), where R is the neutron-star radius) is estimated to lie in the range 1.3×10^{-3} s ($m=3$) to 3.4×10^{-2} s ($m=5$), by using the growth times calculated for the non-rotating models¹¹ and their angular-velocity dependence^{12,13}. We expect that, at present, $d^{n+1}F/dr^{n+1} \approx (-2/\tau_v) d^n F/dr^n$ for $n \geq 1$, where the approximately constant effective damping rate is $1/\tau'_m = 1/\tau_v - 1/\tau_m$.

The expectation that $\tau_m \ll \tau_v$ and the fact that $\tau_m \ll 1$ year if the neutron star was born with its maximum uniform rotation rate leads to the following evolutionary scenario. The amplitude ΔR of all such modes built up quickly (on a timescale of the order of τ_m) until limited by nonlinear effects to a value $\leq R$. The star then spun down rapidly (with constant ΔR but increasing timescale τ_m) until $\tau_m > \tau_v$. At present, $T_0 \gg 0.5$ yr $> \tau'_v$. If τ'_v were larger, the slow-down rate would exceed the observed limit, unless $\tau_v \geq 10^3((\Delta R)_{\max}/R)^2$ yr. If the present temperature of the neutron star is about 10^9 K, we estimate that the viscous timescale τ_v is of the order of 10^6 s if dominated by neutron-neutron scattering (in the absence of magnetic effects)¹⁴⁻¹⁶.

In the phase through which the neutron star is presently evolving,

$$\left(\frac{F(t)}{F_m} - 1\right)^{-2m} \approx \left(\frac{F(0)}{F_m} - 1\right)^{-2m} + \left(\frac{mQ_m\tau'_v}{\tau'_m}\right)[1 - \exp(-2t/\tau'_v)] \quad (2)$$

Here, Q_m is an approximate constant of the order of $((\Delta R)_{\max}/R)^2$. Although this evolution depends on both the gravitational-growth and the viscous-damping timescales, its rate is controlled mainly by the latter, as the former is somewhat greater at present. From equation (1) we note that if $\tau_m \approx 0.1$ yr now, the present limit on the slow-down time T_0 places an upper limit of ~ 0.01 on $\Delta R/R$. Should an evolution of the frequency similar to that predicted by equation (2) be observed, a good estimate of the value of τ_v , which is poorly known at present, could be derived.

All other competing sources of spin-down, such as the usual one due to electromagnetic torques, yield a slow-down time T_0 of at least 10^7 yr. This model-independent value (assuming a moment of inertia $I \approx 10^{45}$ g cm²) follows from the limit of $\dot{E} \leq 3 \times 10^{38}$ erg s⁻¹ on the total power emitted by the pulsar in the form of electromagnetic radiation or charged particles, obtained from the bolometric luminosity of the supernova. Accretion torques provide a source of spin-up. If the mass accretion rate is constrained by the Eddington limit (which happens to match the above limit on $\dot{E}$), the accretion timescale is then also at least 10^4 times greater than the observed limit on T_0 .

Even such a high accretion rate limits the surface value, B , of the magnetic dipole field to no more than about 10^9 G. A stronger magnetic field (or a lower accretion rate at this field value) would lead to the expulsion of the accreting matter beyond the light cylinder. But for a free pulsar the same limits on $\dot{E}$ also give^{1,7} $B < 10^9$ G. If the values of B and the accretion rate $\dot{M}$ are such that the Ghosh-Lamb radius is comparable to the light cylinder radius (2.4×10^6 cm) — for example, if $\dot{M}$ is near the Eddington value and $B \approx 10^9$ G — it is possible that the pulsar turns on and off intermittently. This could explain the lack of pulsations in later observations¹. The optical emission could arise in the free pulsar phase or in the X-ray (accreting) pulsar phase. Finally, our rotational interpretation of F requires the mean density of the star to be at least five times that of nuclear matter.

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Received 22 February 1989.

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Radiation limits

SIR—If, as John Dunster dubiously argues¹, “latency” is to be regarded as a crucial factor in setting permissible radiation doses, then standards should be set to protect the youngest members of society, as they have the longest latency period and the maximum ‘detriment’. Unfortunately, the youngest are also the most radio-sensitive. Thus the excess relative risk of all cancers except leukaemia for those survivors who were under 10 years old at the time of the atomic bombings is about eight times higher than it is for those who were 35 or over². Furthermore, the doubling

dose for leukaemia in children under 10 at the time of the bombings is only 80 mSv. Exposure *in utero* may be even more hazardous; data from obstetric radiography indicates a doubling dose for all childhood malignancies as low as 10 mSv^{3,4}.

The logic of such observations is severely to tighten public dose limits. The International Commission on Radiological Protection (ICRP) has recommended that lifetime exposure should not exceed 1 mSv per annum, but the UK legal limit is still 5 mSv. The National Radiological Protection Board has recommended 0.5 mSv per annum⁵, but has since increased its estimate of cancer risk for the general population to 4.5 times the ICRP figure of 1 death per 10,000 per 10 mSv⁶. In the United States the public dose limit has been 0.25 mSv for the past 10 years. Dunster’s call for a ‘measured response’, and ICRP’s reluctance to revise its own system of dose limitations⁷ are not supported by the scientific data. A legal limit of 0.2 mSv per annum is urgently needed.

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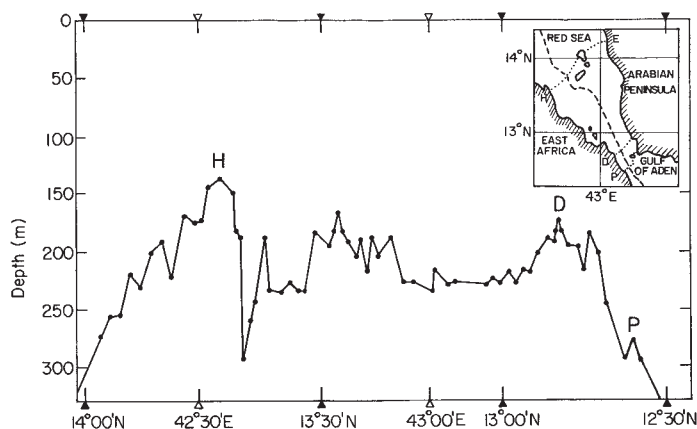
Red Sea salinity

SIR—Thunell *et al.*¹ estimate the palaeo-salinity of the Red Sea for three different sea-surface levels: 80 m, 120 m and 150 m below the present-day level, based on strait-dynamics considerations², and show that their results compare favourably with palaeo-salinity estimates based on δ^{18} O of foraminifera. But they fail to take into account the fact that below a certain sea level, there will be a change in the sill responsible for flow control.

The strait of Bab-el-Mandeb, connecting the Red Sea with the Gulf of Aden (insert in figure), is a long strait, in the dynamic sense³, and contains two main sills (see figure); the Hanish sill, at about 13°40' N, is shallow and wide, whereas the Dumeira sill, at about 12°50' N, is deeper but narrower. From strait-dynamics calculations², taking width and depth each with its prescribed weight, it is readily shown that at the present-day sea level, the Dumeira sill dominates the flow, and the Hanish sill has only a secondary effect on flow control.

Thunell *et al.* do not take into account that, with a sea-level drop of 70 m or more, the Hanish sill would take over the

Bottom depth of the southern Red Sea between the Gulf of Aden and 14° N, at 60 selected points along the main trough (dashed line in insert). H denotes the Hanish sill, D denotes the Dumeira sill, and P the Perim sill. The total distance between the two main sills H and D is 125 km. Full triangles on the upper and lower frame-lines indicate latitude lines crossing the main trough, and open triangles indicate longitude lines crossing the main trough. Insert: a map of the southern Red Sea showing the location of the main trough (dashed line), and three sill cross-sections (dotted lines).



control of the flow. At a level drop of 120 m, their estimated sill depth of $D=60$ m fits the geometry of the Dumeira sill, but the relevant width and depth are those for the Hanish sill, $W \approx 7$ km and $D \approx 30$ m respectively.

For a 120-m sea-level drop, therefore, and retaining their values of evaporation rate and strait length (Q and L , respectively, in their notation) and their assumption that bottom topography was the same as that at present, application of their equation (4) would yield a supersaturation salinity ratio $R=0.1$, rather than the moderate value of $R=0.75$ proposed by Thunell *et al.* Therefore, their conclusion that there is a good agreement between the two independent methods is not valid.

In regard to their estimates for a 150 m level drop, the Hanish sill would in this case be totally exposed, and so the Red Sea would cease to be connected to the world's oceans. Sea-strait dynamics considerations would then not be applicable.

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THUNELL REPLIES—Anati maintains that with a sea-level drop of more than -70 m, flow through the Bab-el-Mandeb strait would be controlled by the Hanish sill rather than the Dumeira sill. He concludes that the Red Sea would have been supersaturated during the last glacial if sea level was lowered by -120 m, and thus that the isotopically derived palaeo-salinities of Thunell *et al.*¹ would not agree with those predicted from the mixing model².

As pointed out by Thunell *et al.*¹, estimates for the magnitude of the sea-level lowering during the last glacial range from -80 to -150 m (ref 4). For a sea-level drop of -80 m, the width and depth of the Hanish sill would be approximately 7 km and 70 m, respectively. Using the glacial evaporation rate and strait-length values reported by Thunell *et al.*¹, a salinity ratio of $R=0.71$ is estimated for an -80 -m sea-level drop. This salinity ratio yields a

palaeo-salinity estimate of 51.7‰, which is consistent with the isotopically derived palaeo-salinity estimates of 48.6 and 50.1‰ (ref. 1).

Thus, for a sea-level drop of -80 m, there is good agreement between the two independent methods of calculating glacial salinities in the Red Sea. These results may also suggest that the effective sea-level lowering in the Bab-el-Mandeb strait region during the last glacial was closer to -80 m than to -120 m.

Note added in proof: Assaf⁵ also used a glacial sea-level lowering of -80 m in the overmixing model¹ to examine water exchange through the Bab-el-Mandeb strait.

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Cowbird song

STR—West and King¹ claim that a wing-stroke display given by captive female brown-headed cowbirds (*Molothrus ater*), in apparent response to 1.1% of male songs, influences male song development. To confirm that an individual responds to a signal, it is necessary to manipulate the proposed signal. Instead of manipulating the female wing stroke directly, West and King found that the seven songs following wing-stroke songs were very potent in eliciting copulatory postures when played later to females, a result they assume to demonstrate that males “immediately” increase the potency of songs in response to wing strokes. But these results would also have been obtained had males ignored female behaviour and simply clustered their potent songs in time. West and King state that males also respond to wing strokes by approaching and inspecting

females but present no data other than pictures of one event.

Other explanations for their observations are that males approach females after potent songs irrespective of the female's behaviour; or that the putative female feedback system is an artefact of captivity. Confining birds for long periods may attune them to the behaviour of cage mates to a greater extent than would be the case for conspecifics in nature. Furthermore, West and King isolated most males from others for six months or more^{1–3}. Males in nature occur together throughout the year, and male–male interactions influence song development⁴. The unnatural deprivation may have made them abnormally receptive to female behaviour. Another artefact relates to the vocal behaviour of captive cowbirds. As well as the ‘perched song’ considered by West and King, wild cowbirds give an acoustically distinct song, the ‘flight whistle’⁵, which is not often sung by caged birds. This is probably because it is usually used for long-distance communication except when the male is close to a female immediately before copulation⁵. West and King¹ say that the perched song is “. . . a defining feature of the release of the copulatory posture”, but about half of the observed copulations in nature, including six involving the sub-species studied by West and King, were not preceded by perched songs⁵. Also, in contrast to the 1:1 sex ratio used by West and King, males in nature outnumber females by at least 1.5:1 (ref. 6). Given this excess of males, a female might find one whose song already conforms to her preference rather than tutor one whose song does not.

West and King suggest that the female guidance occurs during winter and spring^{2,3}. Cowbirds often winter in large flocks, migrate long distances and show extensive mixing of different breeding populations⁷. It is unclear why a male would alter his singing to suit a female with whom he is unlikely to associate during the height of the breeding season. Calling the wing stroke a display, as West and King do, is premature; a display is a behaviour that evolves because it transmits information beneficial to the sender⁸. We see no clear adaptive reason for a female to tutor a male, particularly because the only demonstrated effects of female ‘guidance’ involve captive males stressing perched songs typical of the females’ subspecies rather than their own^{2,3}. Yet West and King have previously suggested that these subspecific song differences function in reproductive isolation⁹.

Finally, West and King may overstate the novelty of their findings. Males develop a range of perched songs without female influence². If female wing strokes in response to certain songs are a precursor of the copulatory posture¹ (given perhaps as a reflex), males could simply

stress those perched songs they find most effective. This is no more novel than showing that individuals with several innate feeding strategies stress the one that results in the most food. It would be novel to show that nonbreeding females in nature purposefully guide male song development, but West and King do not do so.

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WEST AND KING REPLY—When Rothstein *et al.* consult the chronology of published work on female social influence in cowbirds¹⁰⁻¹⁷, they will find answers to many of their questions.

Two inaccuracies need to be clarified. First, North Carolina cowbirds are on breeding habitats in March and April, court in March and lay eggs in late April; 30-50-day-old juveniles are collected at our laboratory in June. The reference on winter habitats cited by Rothstein *et al.* includes no records from North Carolina and conflicts with other reports for the species¹⁸. We regret that our report¹ did not state that all birds were from North Carolina. Intraspecific differences in vocal behaviour in cowbirds are potentially relevant to understanding the influence of ecology on ontogeny¹⁹⁻²¹.

Second, Rothstein *et al.* misunderstand us when they introduce the topic of flight whistles. We wrote that copulatory postures and wing strokes both occur while the male's song is in progress. We use the criterion of temporal co-occurrence to restrict analysis to objectively proximate effects. If females adopted copulatory postures or wing stroked during a flight whistle, we would define it as a response to that signal. We did test a flight whistle (song number 8 in experiment 1): no female responded.

We have also tested Rothstein's hypothesis that a flight whistle serves as a final check on a male's identity before mating. Field recordings of four local and two distant (240 km) distinct types of flight whistle, and six local and distant song types, from breeding *M. a. obscurus* males in Texas were played to five females from the same site using the methods described¹. The females responded to a mean of 9 per cent (range 3-16 per cent) of the flight whistles and 55 per cent (range 37-87 per cent) of the songs. No evidence of flight whistle selectivity was present; the most distant flight whistle received the most responses (16 per cent).

We do not dispute that cowbirds in the western United States may behave differently; we predict geographical differences on empirical grounds²¹. But the relevance of the remarks to the issues at hand is tenuous. Our report describes a behav-

oural means by which non-singing females could affect vocal behaviour in males. Multiple lines of evidence led to the postulation of social shaping, including data from North Carolina populations suggesting that females benefited from such behaviour in terms of enhanced ability to assess males' singing ability, and that males benefited by configuring maximally effective repertoires^{13,17}. The data on wing stroking provide a concrete example of how shaping could occur. The data may not be persuasive to those who consider studies of animals in captivity artefactual. But, for those who share our interest in understanding social and cognitive capacities underlying vocal learning, the data introduce a new level of inquiry — visual experience — and a new form of vocal learning — non-imitative social shaping — findings with widespread implications for behavioural and neurobiological studies of songbirds.

Nevertheless, we have not ignored the issue of relevance to natural conditions. We studied the rate and stages of vocal development in captive and wild North Carolina males, finding no differences¹⁵. Eastzer²² also used our laboratory data on geographical differences in playback responsiveness to explain structural variation in vocal behaviour in eight field populations in two subspecies.

Rothstein *et al.* also question whether wing stroking produced enduring effects. Such an effect is not necessary to show ontogenetic influences²³. We can add, however, that the wing-stroke songs tested were the most potent and most

frequently produced song type during the breeding season for the six males on which data were available. Furthermore, differences in the vocal phenotypes of males raised with females first appear in late autumn suggesting that long-term deprivation from males is not needed to direct a male's attention to female behaviour¹⁵.

Finally, whether or not the wing stroke is a display is controversial^{18,24,25}. The novelty of the work is that it advances research on birdsong into new sensory and social domains. To suggest that novelty cannot be claimed until we show that females "purposefully guide" behaviour represents a retreat from modern principles of behavioural analysis. With C. Lloyd Morgan's 1894 canon²⁶ as a guide, we aspire only to the investigation of the role of experience in changing behaviour.

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First come

SIR—Carilli, van Gorkum and Stocke¹ have recently shown that neutral atomic hydrogen observed in both absorption and emission extends from NGC 3067 in the direction of the QSO 3C232 and apparently completely envelopes it. The obvious conclusion, which they never directly make, is that this provides direct evidence that the two objects are physically associated despite the fact that their redshifts are very different.

What these authors do not point out, though they do reference the paper in another connection, is that this quasar-galaxy pair was one of four pairs shown in 1971 by myself and colleagues² to be much closer to bright galaxies than would be expected by chance among all 47 of the 3C and 3CR quasars identified at that time. This result was farther confirmed by Monte Carlo calculations made by Kippenhahn and de Vries³.

Thus we concluded in 1971 that those pairs were physically associated. The new result¹ clearly supports this conclusion and adds further to the unpopular but real evidence that the redshifts of some quasars, at least, are not measures of their distances.

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Articles of faith

Fred Hoyle

Science and Providence: God's Interaction with the World. By John Polkinghorne. SPCK, Holy Trinity Church, Marylebone Road, London NW1 4DU: 1989. Pp.114. Pbk £5.95.

It is probable that everybody is infused with some sense of religion. Scientists have a belief in a world with order. Some scientists even have a half-formed belief in a world of purpose, a view held overwhelmingly by the majority of people. Over the millennia, attempts have been made to understand what such a purpose might be. Yet despite the expenditure of a great deal of time, energy and emotion, nothing has emerged in this matter in a clear light of rational thought such as we understand it in science.

The procedure adopted in all religions is to postulate the existence of an entity or entities with a full understanding of the purpose of the Universe, and of all the other emotional matters that we see only very darkly. To make use of this postulate, communication between human beings and the entities has to be established in some way. It is the manner in which this is supposed to have been done that distinguishes the different religions. It can be done through holy men or prophets, who are supposed to be endowed with special properties, as in Judaism and Mohammedanism. It can be done through the entities taking on human form, as in Greek and other still older religions. And as Christianity demonstrates, it can be done through the symbiosis of a unique phenomenon that is part-human, part-entity. Furthermore, it is crucial to the survival of a religion that the communication which is supposed to have been achieved in one or other of these ways be set down in a document, to which frequent references can be made as time passes. It was largely because the older religions did not satisfy this last condition that they have not survived.

The approach is necessarily self-contradictory because it is a pretence that problems have been solved which have not been solved, or indeed which may even be illusions or not real problems at all. Two ways of dealing with the contradictions have been adopted. By far the most successful, in terms of numbers of satisfied adherents, is the fundamentalist method of forbidding all mention of the contradictions. The alternative is to argue matters around and around in the hope that somehow the contradictions will disappear. It is this alternative that in Christian belief distinguishes the Protestant from the Catholic. Matters have been argued around and around in Protestantism from Martin Luther to the modern

Anglicans — and in the case of the present book, to John Polkinghorne.

The book consists of nine loosely jointed essays, in the first and seventh of which the physical nature of the entity, God, is considered. Polkinghorne does not like the modern trend among some scientists for regarding God as the designer of physical laws and nothing else. For this would make God too remote, too distant from the affairs of individual people. To

I came to this question was in the second essay where Polkinghorne describes what the late J. E. Littlewood used to call the hook and slice effect — namely, that in an initial value problem an exceedingly small change of the boundary conditions can sometimes produce a large change in the outcome. So one might suppose that, with only small perturbations, God could produce miraculous happenings, although how water could be changed to wine in this way is quite unclear. I had the impression that Polkinghorne, while seeking to defend miracles in various ways, would be glad to be rid of them, with the exception of the resurrection. Polkinghorne accepts the resurrection, literally it seems, on the grounds that it "is readily accommodated in Christian theology". Of course it is. But to argue this way is tautological.

It seems to me that Polkinghorne is at his best in the chapter on "Evil". The Augustinian dilemma that God cannot be both all-powerful and all-good is well known. To his considerable credit, Polkinghorne does not accept the easy resolutions of the problem of 'evil' which have been proposed by people who never experienced it. For example, he quotes with approval the devastating comment — "No [promise of] Heaven can rectify Auschwitz".

Most of the usually quoted acute examples of 'evil' can be seen to be derivative from a broader all-embracing evil, the evil of decay, the evil that in three score years and ten brings low all human achievements, however great they may be. The situation is well expressed by the saying "Those whom the Gods love die young" — presumably with the corollary that those whom the Gods hate live to a great age.

Nature's resolution of the problem of decay lies in DNA and in the details of biological reproduction. By a dazzling sequence of hugely complicated steps the whole system of life regenerates itself. The best is replaced by the best, or even better. If a higher entity is involved in this arrangement, it looks, on a commonsense view of the matter, as if the entity is not much concerned with the fall of individual sparrows. Either that or the entity is not all-powerful. The situation, as we find it, is the best that has been achieved to date, a view close to that of Leibniz. While the worst contradictions of religion would be avoided by this position, the necessary demise of a personal God is not welcomed. Religion is not so much a search for a deeper understanding of the world as a search for a validation of a pre-set emotional position. Christians do not enter arguments of the kind discussed in Polkinghorne's book on the understanding that they may be forced to abandon some or all of their beliefs. A pre-set condition of the argument is that they must not lose, which makes such disputes pointless,

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REASONS

John Polkinghorne — seeing God in three and four dimensions.

obtain a God who sees every sparrow fall, Polkinghorne in his seventh essay takes what for me was the surprising step of associating God with a three-dimensional spacelike section of the Universe, as if God were identified with a set of Cauchy conditions on partial differential equations governing the Universe. Then, recognizing the need for God to be all pervasive, He is also endowed with four-dimensional cognizance of the Universe, a contradiction which for Polkinghorne seems to be resolved by describing God as "dipolar", a word which I fear did nothing for me.

I was led to read this book by its subtitle, by the prospect of being told how God interacts with the world. The nearest

uninteresting and in some degree distasteful to the outsider.

The stupendous complexity of the way in which biological systems have overcome decay has always seemed to me an attractive point of attack on the problem of teleology. I have been much criticized for holding the view that explanations offered by standard biological theory are facile. But that is exactly what they are — facile, inadequate ideas that are accepted and believed in because of a fear of the deeper issues that might otherwise come to light.

Science has been very ready to destroy religious beliefs, without trying very much to offer anything emotionally satisfactory to society in return. So long as science was producing in material terms far more than it consumed this was all very well. But today science is not producing a great deal more than it consumes, especially in physics, with the consequence that there is talk already of shutting down physics in half the universities in Britain. Where physics goes it seems unlikely that the rest of science can avoid following. By eschewing issues that most people feel deeply about, science has produced a situation in which it has few friends outside itself. Polkinghorne may turn out to have far more. □

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New journals review

THIS year *Nature's* annual new journals review supplement will appear in the issue of 28 September. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals which first appeared after June 1987, and which issued at least four separate numbers by the end of April 1989, will be considered for review.

- Journals covering any aspect of science are eligible, though those dealing with clinical medicine, engineering and pure mathematics are excluded (as are abstracts' publications).

- Frequency of publication must be at least three times a year.

- The main language used must be English. Translation journals in English are of course eligible.

Those submitting journals for review should promptly send at least *four different issues* (the first, the most recent and any two others) of each title to: Tim Lincoln, *Nature*, 4 Little Essex Street, London WC2R 3LF, England.

For further information please telephone Tim Lincoln on 01-836-6633 (011-44-1-836-6633 from the United States), extension 2414.

Way out of the waste land

Robert W. Cahn

Radioactive Waste Forms for the Future.

Edited by Werner Lutze and Rodney C. Ewing. North-Holland: 1988. Pp.778. Dfl.470, \$247.25.

WHEN one reads a diatribe against nuclear power, one often finds the bald assertion that no way exists of disposing of radioactive wastes: as long as nuclear reactors are allowed to exist, wastes must relentlessly accumulate, we are told, and finish up by killing us all. Those who detest and are afraid of nuclear power echo the poet and critic William Empson:

Slowly the poison the whole blood stream fills.

It is not the effort nor the failure tires.

The waste remains, the waste remains and kills.

The contributors to this book show, in exhaustive detail, why the waste remains and does not kill.

When a charge of nuclear fuel is 'spent', it is discharged into cooling ponds so that its radioactivity can diminish over a period of a few years to a level which permits safe remote reprocessing treatment. The remaining radioactive fission products (high-level wastes) can then be separated out and concentrated in solution; many thousands of litres have been stored for some years in this form while the best long-term strategy for permanent disposal in solids was being developed. Such a strategy is now generally agreed and has been put into effect in many countries. The assertion that no such method exists is simply untrue.

The editors claim that this is the only book which systematically pulls together the enormous amount of work done over the past 30 years on the alternative ways of 'immobilizing' high-level nuclear wastes. Much of that research has been published in the 'grey literature' of final reports from various national laboratories or in conference proceedings, many of them not easily accessible.

The procedure which is now in large-scale operation in France, Belgium (in a plant built by Germany) and India, which is about to start large-scale operation in the United States, Britain and Japan, and which operates at pilot-plant scale in Canada and Italy, is vitrification; that is, the incorporation of the waste within blocks of borosilicate glass which are then further encapsulated in welded metallic containers, for later burial in deep tectonically inert caverns (initially in reversible disposition, later in permanently sealed sites). The principle of multiple

barriers is crucial to this strategy: the glass itself, the outer container, the inert locations all have their parts to play. A number of other strategies have been the subject of much experimental attention, most of them involving various forms of ceramic vehicle instead of glass (notably the Australian Synroc and the American tailored ceramics). All of these are covered in the book, which however does not discuss the geological character of suitable burial sites; this topic has been covered elsewhere.

Ten chapters cover the various types of immobilization, the most comprehensive of them being Lutze's 160-page survey of borosilicate glasses. There is also one Canadian contribution which discusses the case for leaving spent fuel unprocessed. A twelfth chapter, by the editors, compares the glass option with the others, primarily with Synroc. This splendid essay forms an appropriate conclusion to the book.

The editors insisted upon a standardized chapter format which would ease critical comparison between the various options. Not only are all the obvious themes covered, such as leaching behaviour, production methods, response to radiation damage and radioactive self-heating, and physical and mechanical characteristics, but evidence is also adduced from natural analogues (such as basaltic glass and palagonites) which allows the long-term chemical stability of man-made materials to be estimated by comparison. Each contribution was scrutinized by a band of 39 reviewers from nine countries, and apparently several of the chapters were extensively revised as a consequence.

A reading of the book makes it clear why, in spite of a plethora of options, all countries which have built or are building solid immobilization plants have chosen the glass route — the amount, variety and reliability of the accumulated information about this system, including production methods, exceeds that about its competitors; and although other systems have some points of superiority (for example, Synroc's lower solubility in ground water), in other respects (for example, smallness of volume change on irradiation) glass scores unambiguously. Nevertheless, as the editors point out, the availability of a number of "fairly thoroughly developed [alternative] waste forms" constitutes an "excellent situation".

This is a critical and unemotional overview of a very large body of information. It will be essential reading both for scientists working in the field and for others who wish to arrive at a fact-based judgement on the degree of danger associated with high-level wastes. □

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An open mind?

John C. Marshall

From Neuropsychology to Mental Structure. By Tim Shallice. Cambridge University Press: 1989. Pp. 462. Hbk £40, \$59; pbk £15, \$24.95.

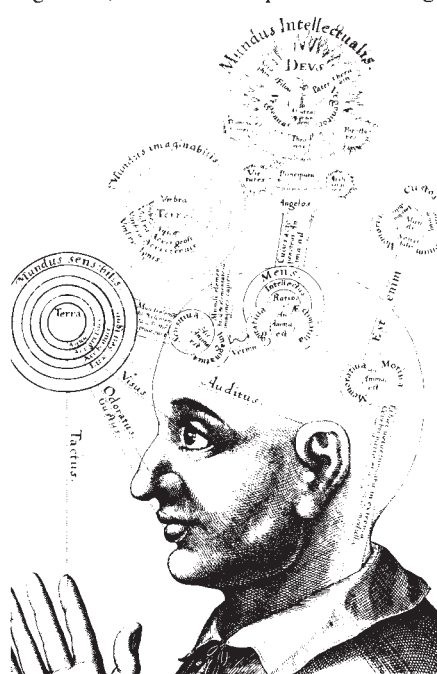
IN HIS masterwork, *An Introduction to the Study of Experimental Medicine* (1865), Claude Bernard recorded his methodological, empirical and theoretical attempts to transform physiology into an exact science. One cornerstone of Bernard's philosophy was his insistence that studies of normal and pathological phenomena were but two sides of the same coin. The point may seem obvious now, yet it was not always so; indeed, there remain areas of medical observation where even today the dictum is not always taken as axiomatic. Neuropsychology is an excellent case in point.

As Tim Shallice states, the discipline seeks "to assess the crippling problems in thought, memory, and language that can occur from brain damage". In an age when strokes, dementing diseases and road traffic accidents (to say nothing of wars) produce an ever-increasing population of cognitively impaired individuals, the potential practical significance of neuropsychological investigations can hardly be overestimated. I write 'potential' because it is a regrettable fact that, although not totally devoid of effect, efforts to rehabilitate the brain-damaged patient do not (yet) constitute a compelling success story. There is, in my opinion, a reason for this (over and above the undoubted truth that discovering how the mind-brain operates is very, very difficult).

In large part, cognitive rehabilitation has been an *ad hoc* business, based upon no firm understanding of the functions that are impaired. And this has in turn been consequent upon a historical divorce between students of normal and of pathological cognitive functioning. For 50 years, the mainstream of neuropsychological inquiry has been driven by the construction of test-batteries, the primary purpose of which has been to assess whether (and to what extent) a patient is impaired in particular domains. With a few notable exceptions, the elucidation of the underlying structures and functions that mediate normal performance has not been undertaken by those whose main concern is the diagnosis of overt deficit. Yet observed disorder after brain damage sustained by a previously healthy adult must reflect (directly or indirectly) loss or distortion of normal mechanisms. We are unlikely, then, to understand the one without trying to understand the other. For Claude Bernard, physiology, pathology and therapeutics was an unbreakable

troika. And so it should be in neuropsychology, with the substitution of psychology for physiology.

I have allowed myself this somewhat lengthy preamble because it is important to be clear about what Tim Shallice is attempting in the book under review. The title — *From Neuropsychology to Mental Structure* — is chosen with care. The text can be read as an advanced introduction to what is currently known about the symptomatology of disorders of long- and short-term memory, knowledge representation, language (including reading and writing skills), visual object recognition, spatial cognition, attention and problem-solving.



Fludd's 1619 diagram of the psychological faculties. From *An Illustrated History of Brain Function*, by Edwin Clarke and Kenneth Dewhurst (Sandford, Oxford, 1972).

But it *should* be read as a detailed theoretical meditation upon how to engage in the intellectual enterprise of cognitive neuropsychology; all the empirical examples of pathological performance are introduced primarily in order to wrestle with the problem of how to make meaningful connections between neuropsychological data and the structure of the (normal) mind-brain. That such a book could be written at all is a tribute to the many scientists who have contributed to the rapid development of the field over the past decade. But it is to Shallice that we owe our gratitude that the first text on theoretical neuropsychology has been done so well.

The basic data of neuropsychology consist of patterns of association and dissociation of symptoms between and within patients. But inference from such observations to the functional architecture of the brain is fraught with problems. Even the most elementary manifestations are subject to occultation. Take the standard example: if a valve is removed from

my radio, which then emits a high-pitched shriek, it does not (necessarily) follow that valves are shriek-suppressors — but they could be (if we knew as little about radios as we do about brains). Consider a patient who reads 'pint' to rhyme with 'hint' (the regular pronunciation of 'i' in this context). Can we infer that the patient has 'lost' a component of the reading system that recognizes printed words as 'wholes' and has been forced to fall back upon a sublexical ('phonic') strategy? On this interpretation the 'whole-word' recognition device *is*, in one sense, an error-suppressor, analogous to a valve being a shriek-suppressor.

The neuropsychological literature abounds with such examples of highly restricted deficits; a patient may, for instance, define abstract words well ('pact' = 'friendly agreement') but reliably fail with concrete words ('poster' = 'no idea'). Does the existence of discrete classes of failure or error imply that there is punctate localization of memory stores for different types of information? Alternatively, could behaviourally circumscribed disorders be simulated by the parallel distributed processing (PDP) systems that are currently so fashionable? This latter question is in turn relevant to debate about whether anatomical data (for example evidence about lesion location and extent) can constrain theories expressed at the level of functional information-processing models. The fact that diffuse lesions can result in cloistered overt deficits must surely be trying to tell us something. For the most part, what Shallice calls 'ultra-cognitive neuropsychology' has proceeded in blithe, albeit principled, disregard of neuroanatomy. Shallice rejects this hard-line position but concedes that "To hope for an advance in theories of the functional organization of cognition by paying special attention to issues of localization is not, at present, a promising strategy". However, in contrast to the majority of scholars who have argued for the relevance of neuroanatomy, Shallice actually provides some examples where knowledge of the nature of a lesion might serve to help adjudicate between rival functional theories.

Shallice's discussion of the logic of single-case studies is likewise exemplary. Claude Bernard argued that the use of group averages in medicine and physiology "leads necessarily to error". And he quoted in support a wonderful study in which a physiologist "took urine from a railroad station urinal where people of all nations passed, and who believed he could thus present an analysis of *average* European urine!". The philosophers of 'ultra-cognitive neuropsychology' have shared Bernard's startled amazement that anyone could seriously report the mean performance of a group of aphasic or agnostic patients on a particular set of

tasks. If we have learned anything it is that only the fine detail of a patient's performance suffices for model building, and that, at this level, theoretically important individual variation is paramount. Shallice does not dismiss all group studies in neuropsychology, but he does give good guidelines concerning their restricted use. In the course of so doing he also attacks one of the sacred methodological cows of the ultra-cognitivists. It is not the case, Shallice argues, that a double-dissociation between two patients' performance on two tasks always implies the existence of two distinct processing modules.

I have touched on only a small sample of the topics that Shallice considers, and should accordingly note that he discusses in depth most of the salient conceptual issues and controversies in the field. Neuropsychologists and behavioural neurologists will need to study this book with pencil in hand and work through the structure of each argument. Man is by nature lazy and inclined to data-collection for its own sake, but the practice of thinking may cure these faults. □

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Language of life

Jonathan Howard

Macmillan Dictionary of Immunology.

By Fred S. Rosen, Lisa A. Steiner and Emil R. Unanue. Macmillan, London/Stockton, New York: 1989. Pp.223. Hbk £25, \$50; pbk £9.95, \$20.

PERHAPS nineteen eighty nine will be remembered as the year when the new edition of *The Oxford English Dictionary* was published. Certainly, anybody obliged by tradition or poverty to put up with any previous Oxford dictionary was living in a lexical world so antiquated as to be disabling for contemporary life.

But £1,500 is a lot to pay for a work that still lacks most of the entries in Rosen, Steiner and Unanue's *Macmillan Dictionary of Immunology*. My mother, one of many people who sent material for the new *OED*, contributed a quarter of a million items, but none of them was immunological. And this despite repeated attempts to convince her that even immunology's ugliest coinage was as worthy as the words she mined from the "trash" (as *The Guardian's* dictionary critic put it a few weeks ago) that she so much liked to read. Well, that's the *OED* for you. Apparently it was a matter of policy not to attempt to grow with scientific language; too much, too fast. Possibly, did they hope, too ephemeral? But scientific language, like the rest of language, is here to be understood and used, and then to pass gracefully into history when its usefulness has been exhausted. And to my mind, at least, it would be sad if there were no record of its passing.

Rosen, Steiner and Unanue's dictionary is excellent. The three authors are immunologists of unimpeachable distinction in a suitably complementary collection of fields (clinical immunology, immunochemistry and cellular immunology). They have done a fine job of covering the whole field, and most impressively of all, have managed to keep the whole thing so up to date that it is actually useful.

One of the more attractive aspects of the definitions is the insistence that much is not yet known. The importance of genetic manipulation techniques to immunology is reflected in entries such as 'cosmid', 'Cot' and 'nick translation'. So much so, perhaps, that 'germinal centre', 'medial histocompatibility antigen' and 'high endothelial venule', all three more distinctively immunological, one would have supposed, appear to have been squeezed out. But if an entry is there, and it often is, it is correct, succinct and right up to the minute. And if HEVs did not make it, the recirculators amongst us will be glad to see that MEL14 is there. Rosen's contribution is perhaps the most distinctive: I see his hand in the quite extraordinary collection of immunological disorders our species is subject to, which litter every page.

Scientists are incorrigible wordsmiths, perhaps in part because to name something confers title, though there are, of course, other more laudable motives. Nowadays, it's a free-for-all. New words teem into the world, willy-nilly born, but not by any means all divinely formed. It was not always so. Michael Faraday consulted the greatest wordsmith of all, the man who gave us 'scientist', when he needed terms to deal with the torrent of new ideas generated by the discovery of the electrolytic decomposition of aqueous solutions. So William Whewell sat down and created 'ions', 'electrodes', 'anodes', 'cathodes' — a whole vocabulary. But who will do for us now what Whewell did for Faraday? 'Cosmid' is a lovely word, 'genome' too; and so are some of the transferred usages such as 'library'. But where was Whewell when 'agretope', 'desetope', 'histotope' and 'restitope', all dutifully defined by our poor authors, first wrinkled our brows? When this dictionary moves into its next edition, I sincerely hope that these immunological oddities will have passed, like 'oecotaxis', into immunolexicographical history. □

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Rocking in the laboratory

Gordon M. Biggar

Simulating the Earth: Experimental

Geochemistry. By J. R. Holloway and B. J. Wood. Unwin Hyman: 1988. Pp.196. Hbk £25, \$39.95; pbk £11.95, \$19.95.

ENCOURAGING the more traditionally trained geological graduates (or undergraduates) to expand their horizons into experimental geology has never been easy. Now, however, in *Simulating the Earth* we have an enjoyable account of some of the best experimental work that has been done, set out with simple instructional diagrams and sketches, and giving all the essentials, all the encouragement that one could hope for.

Each example tells the story of what the experimenter was trying to achieve and how well he succeeded. Thereby another brick is added to the edifice of geological (physical and chemical) prediction, leaving readers with an understanding of many geological processes which they may have seen in their rocks, sometimes without realizing that they were accessible to experimental verification. If students or researchers are sufficiently inspired to turn their hands to practicalities, there are many well-equipped experimental laboratories around the world which welcome working visitors. Those visitors can now commence their experiments well informed of what they might achieve and how.

For all its modest cost, *Simulating the Earth* is two books in one — a practical guide to the equipment in the early chapters and the appendices, and a comprehensive intellectual guide, often through the examples provided, to the whys and wherefores. Many fields are covered: there is something for everybody, no matter whether 'everybody' is interested in metamorphic rocks, thermodynamics, fluid phases, speciation of ions in fluids and liquids, volatilization, spectroscopy, igneous rocks, element partitioning, differentiation viscosity or many other topics. Nor will established workers in experimental geology be disappointed (the book is subtitled *Experimental Geochemistry*, but moves in Chapter 9 into measurements of physical properties). They will find a concise resumé of what they thought they knew, and some hidden gems of information or philosophy to widen appreciation of their speciality. "Putting it all together", to quote from Chapter 4, is what Holloway and Wood have succeeded in doing, and this phrase summarizes the whole book. □

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The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells

D. Metcalf

Several glycoproteins that control blood-cell production and function have been purified and sequenced. The four colony-stimulating factors interact in a complex way to regulate the differentiation and maturation of the granulocyte and macrophage lineages and have potential applications for the clinical manipulation of blood-cell production.

MOST mature blood cells are short-lived and must be replaced continuously throughout adult life. Blood cells originate from a self-renewing population of multipotential haemopoietic stem cells, located mainly in the bone marrow, which generate progenitor cells committed irreversibly to one or other of the various haemopoietic lineages (see Box). Progenitor cells in turn can each generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells^{1,2}. What mechanisms are involved in achieving the coordinated production of the eight main types of blood cells and how are these critical events of differentiation commitment and cellular maturation regulated?

From studies on cultured haemopoietic cells it is apparent that much of the control of haemopoiesis is mediated by a group of glycoprotein molecules, loosely termed haemopoietic growth factors. Eleven of these have been purified, their genes cloned and active recombinant molecules produced (Table 1). The discussion here will consider only those molecules controlling granulocyte-macrophage populations but, where information is available, the general principles emerging seem to apply equally to the other haemopoietic growth factors.

Neutrophilic granulocytes and macrophages are cells of different morphology that are derived from common progenitor cells; both are involved in the disposal of invading microorganisms or damaged tissue cells. The formation of granulocytes and macrophages can be studied *in vitro* because of the development of semi-solid culture systems in which committed granulocyte-macrophage progenitors proliferate to form clones (colonies) of maturing granulocytes and/or macrophages^{14,15} (Fig. 1). As the cultures are clonal, progeny of individual cells can be analysed, an important advantage because progenitor cells are heterogeneous. Many progenitors can form both granulocytes and macrophages and such bipotential progenitors also offer a simple model for examining aspects of the differentiation commitment process.

Signal molecules and receptors

The proliferation of cells in culture to form colonies of maturing granulocytes and macrophages does not occur unless cells from various tissues such as the lung, heart, spleen and uterus, or medium conditioned by these cells are included in the culture¹⁴⁻¹⁶. Biochemical analysis of material with colony-stimulating activity led to the discovery and purification of four distinct glycoproteins, each able to stimulate colony formation. There are four of these colony-stimulating factors (CSF): granulocyte-macrophage (GM-CSF), granulocyte (G-CSF), macrophage (M-CSF) and multipotential (multi-CSF)². More recently a fifth glycoprotein, interleukin-6, has also been shown to have colony-stimulating activity¹⁷. The cellular sources of the CSFs, their levels in tissues and the blood, and the manner in which CSF production is modulated in response to changing demands are discussed elsewhere¹⁶. Cell types known to produce

one or more CSFs include fibroblasts, endothelial cells, stromal cells, macrophages and lymphocytes.

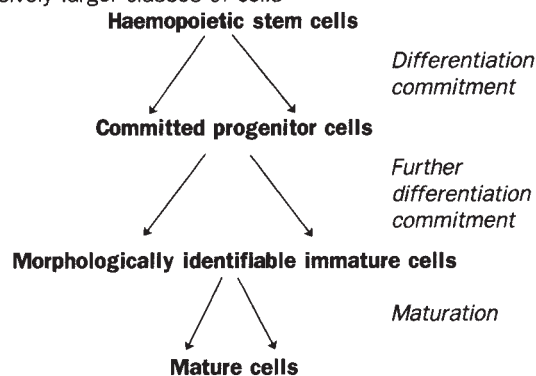
The CSFs have considerable overlap in their proliferative actions on granulocyte-macrophage progenitors as shown schematically in Fig. 2. GM-CSF and multi-CSF can stimulate the formation of both granulocytes and macrophages, G-CSF tends to stimulate only granulocyte formation and M-CSF only macrophage formation¹⁸. Interleukin-6 resembles G-CSF in its actions, at least on murine granulocyte-macrophage cells¹⁹. In spite of their overlapping actions, the CSF polypeptide chains share no significant amino-acid homology and differ in predicted secondary structure. The carbohydrate portions of the CSFs are not necessary for their biological action.

Unique membrane receptors exist for each CSF and are found on granulocyte-macrophage progenitors and their maturing progeny²⁰. An unusual feature is that most cells simultaneously exhibit receptors for more than one type of CSF and the progenitors can respond, at least initially, to proliferative stimulation by any of the four factors. Although one CSF cannot bind to other CSF receptors, occupation by one CSF of its receptors can influence the behaviour of the receptors for other CSFs on the same cell²¹.

Only one of the CSF receptors has been identified, that for M-CSF, which is the *c-fms* proto-oncogene product²². This receptor has an intracellular tyrosine kinase domain and seems to function in a similar manner to the well-studied receptor for epidermal growth factor. At least two of the other CSF receptors

The hierarchy of haemopoietic populations

Haemopoiesis proceeds by the clonal proliferation of three successively larger classes of cells



This pattern is common to all the eight blood-cell lineages. Maturation proceeds in parallel with the final clonal proliferation; the most mature cells, which enter the blood, are post-mitotic and incapable of further division in some but not all lineages.

TABLE 1 Known haemopoietic growth factors and their target cells

Factor	Symbol	Target cells	Ref.
Granulocyte-macrophage colony-stimulating factor	GM-CSF	M, G, eo, meg, multi	3
Granulocyte colony-stimulating factor	G-CSF	G, M	4
Macrophage colony-stimulating factor	M-CSF	M, G	5
Multipotential colony-stimulating factor	Multi-CSF, IL-3	G, M, eo, meg, mast, multi stem	6
Erythropoietin	Epo	E	7
Interleukin-1	IL-1	Stem	8
Interleukin-2	IL-2	T, B	9
Interleukin-4	IL-4	B, T, mast	10
Interleukin-5	IL-5	B, eo	11
Interleukin-6	IL-6	B, T, G, multi	12
Leukaemia Inhibitory Factor	LIF	M	13

All except IL-1 are glycoproteins with similar polypeptide chain (or subunit) size (M_r 15,000–20,000). References refer to the publications describing the cloning of the respective human cDNAs. E, erythroid; M, macrophage; G, neutrophilic granulocyte; eo, eosinophil; meg, megakaryocyte; mast, mast cells; multi, multipotential progenitor cells; stem, stem cells; T, T lymphocyte; B, B lymphocyte.

seem too small to contain tyrosine kinase domains but their signalling mechanisms are unknown²³.

The CSFs are biologically highly active and stimulate cell proliferation at concentrations of 10^{-10} – 10^{-12} M. Most haemopoietic cells have only a few hundred CSF receptors per cell²³ but analysis of CSF-receptor interactions under steady-state conditions indicates that CSFs can stimulate maximal biological responses when as few as 10% of receptors are occupied²⁴.

Proliferative actions of CSFs

The CSFs induce any non-dividing granulocyte-macrophage precursors to start dividing within three hours^{25,26}. CSF stimulation is required throughout the cell cycle and high concentrations of CSFs not only shorten mean cell-cycle times but seem to delay maturation of cells by preventing them from entering the post-mitotic state. This means a higher proportion of progeny remain capable of further division, with the formation of larger numbers of mature progeny²⁷. These observations indicate that the precursor cells are not stringently restricted in the number of cell divisions which they can undergo before maturing to the post-mitotic state. The possibility that the post-mitotic state may be because the cells cease to express growth factor receptors on their membranes is eliminated by the observation that the most

mature, post-mitotic granulocyte (the polymorph) continues to exhibit CSF receptors^{28,29}.

The relationship between CSF concentration and cell-cycle times indicates that the rate of progress of a cell through the cell cycle depends on the intracellular concentration of bound CSF receptors or of key components of the resulting metabolic cascades. A combination of two CSFs might be expected to result in an additive proliferative response, but superadditive responses are observed², implying that enhanced signalling is possible when two or more types of CSF receptor are activated on the same cell.

GM-CSF is unusual because it stimulates the proliferation of a progressively broader spectrum of progenitor cells as concentrations are increased: first macrophage progenitors, then granulocyte-macrophage, followed by granulocyte, eosinophil, megakaryocytic and multipotential progenitors in that sequence¹⁸. This phenomenon occurs in concentration ranges that are likely to be physiological and is also seen when GM-CSF is injected into mice. It may indicate that progenitors in different lineages require higher concentrations of activated GM-CSF receptors before proliferation occurs.

The observed lineage-specificity of a growth factor may largely be determined by the genetic programming of the cell, which dictates which growth factor receptor will be exhibited. The interesting permissiveness evident in the ability of certain growth factors and their receptors to stimulate the proliferation of inappropriate cell types indicates that at least part of the metabolic cascade activated following growth factor-receptor binding may be common to a number of receptors and cell types. When complementary DNA for the M-CSF receptor is inserted into fibroblasts, with the subsequent expression of M-CSF receptors, the fibroblasts will proliferate in response to stimulation by M-CSF³⁰. Conversely, insertion of the epidermal growth factor (EGF) receptor into CSF-dependent cell lines allows them to be stimulated by EGF^{31,32}.

Common signalling pathways do not present conceptual difficulties for explaining the actions of the CSFs, where a single type of response, such as cell division, is involved. Responses reported to be induced by CSFs, such as changes in intracellular ATP levels³³ and glucose transport³⁴, activation of protein kinase C (ref. 35) and the Na^+/H^+ antiporter³⁶, may be the result of shared signals.

Multiple actions of CSFs

The CSFs are not simply proliferative stimuli for responsive cells. *In vitro*, each has four distinct additional actions: enhancement of the survival of precursors and mature cells by maintaining the functional integrity of the cell membrane; irreversible

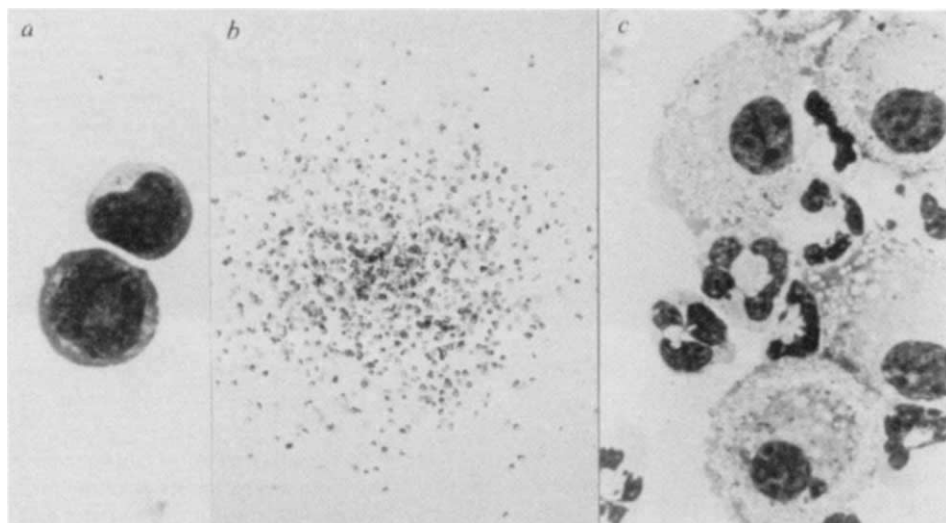


FIG. 1 The main cellular events in haemopoiesis can be monitored using clonal cultures of haemopoietic cells using, if need be, serum-free medium. Unfractionated cells can be cultured or, as shown in (a), haemopoietic progenitor cells can first be purified by fluorescence-activated cell sorting. In semi-solid cultures, individual progenitor cells can be stimulated by purified colony-stimulating factors to form b, colonies of progeny cells. With time, colony cells progressively mature, for example to mature granulocytes (polymorphs) and macrophages (c).

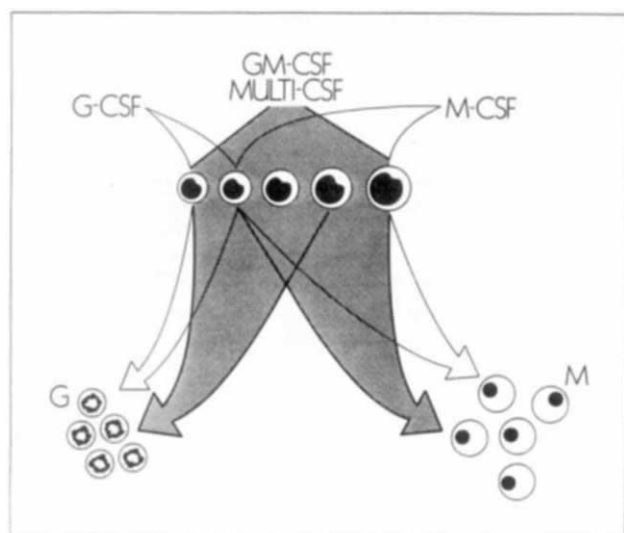


FIG. 2 The colony-stimulating factors overlap in their control of the proliferation of granulocyte-macrophage progenitor cells to form maturing granulocytes (polymorphs) and macrophages. Both GM-CSF and multi-CSF *in vitro* can stimulate most progenitors to form both types of progeny. M-CSF stimulates the same progenitors to form mainly macrophages, whereas G-CSF is a complete proliferative stimulus for only some progenitors, with the formation mainly of granulocytes. Maturing cells continue to express CSF receptors and are functionally stimulated by the CSFs. Commitment to differentiate selectively into either macrophages or granulocytes can be influenced by the concentration and type of CSF that stimulates the initial divisions of the progenitor cells.

induction of differentiation commitment; induction of cellular maturation, and stimulation of the functional activity of mature cells, for example, phagocytosis or the synthesis of a variety of biologically active molecules. When this combination of actions was first described, the concept that a regulatory molecule might have quite diverse actions on cells of a single lineage was novel, but now this pattern is being observed for regulatory molecules acting on other cell types—for example, platelet-derived growth factor enhances both collagen formation and bone calcification³⁷.

The studies with CSF demonstrate that the response observed following CSF interaction with its receptor is dictated by the nature of the responding cell. For instance, GM-CSF stimulates cell division in progenitor cells but enhances the survival and functional activity of post-mitotic granulocytes without stimulating cell division.

At least some CSFs seem to have only a single type of membrane receptor, yet they elicit a wide range of responses, from stimulation of phagocytosis, which probably occurs locally at the cell membrane, to differentiation commitment and maturation, which must ultimately involve complex and selective changes in gene transcription. Reference was made above to the possibility that the proliferative signals arising from different CSF receptors may be of a single type, but the radically differing responses that can be initiated by activating a CSF receptor indicate that the receptors are linked to several signalling cascades, some of which are likely to be specific to a particular type of receptor.

Commitment in normal and leukaemia cells

Commitment of haemopoietic cells to a lineage is irreversible and becomes more apparent with each successive cell division³⁸. The generation of progeny from multipotential haemopoietic progenitors that are committed to single or restricted lineages seems to be random rather than intrinsically determined³⁹, but extrinsic factors can enhance the probability of commitment to a particular lineage. Bipotential progenitors can produce either granulocytes or macrophages, depending on the concentration²⁷

and type³⁸ of CSF used to stimulate the initial divisions; for example, stimulation with M-CSF tends to produce progeny committed exclusively to the formation of macrophages³⁸. In this simple model, the CSFs compete with each other and, if used simultaneously, varying proportions of both types of committed progeny can be generated² (Fig. 3). It seems that, although different CSFs may initiate common metabolic cascades to stimulate cell division, commitment must require unique elements in the signalling cascades.

A potentially similar commitment process can be observed in established myeloid leukaemia cell lines. During division, these leukaemia cells have the alternatives of self renewal, that is replication without commitment, or commitment to the formation of differentiating progeny, but they usually exhibit an extremely high level of self renewal. Self renewal can be suppressed and differentiation commitment induced in such lines by more than one haemopoietic regulator: for example, G-CSF or interleukin-6 for WEHI-3B cells^{40,41}; the leukaemia inhibitory factor, interleukin-6 or G-CSF for M1 cells⁴¹⁻⁴³; G-CSF or GM-CSF for HL60 and U937 cells^{44,45}.

The induction of commitment on leukaemia cells by these factors can have striking similarities to the promotion of commitment by the CSFs acting on normal bipotential progenitor cells⁴⁶. Suppression of self renewal does not always lead to the formation of maturing progeny⁴⁴, so maturation is not a necessary consequence of differentiation commitment.

The ability of some haemopoietic regulators to suppress the self renewal of leukaemic cells highlights a curious contradiction in their actions. The CSFs stimulate proliferation, which necessarily implies an ability to encourage self renewal, or at least postpone maturation into postmitotic cells; acting on other cells, the same CSFs can effectively suppress self renewal and induce differentiation.

A more dramatic example of these opposite actions is seen with the leukaemia inhibitory factor, a potent suppressor of self renewal in M1 leukaemia cells⁴². At the same concentrations, leukaemia inhibitory factor acting on totipotent normal embryonic stem cells suppresses differentiation commitment, which otherwise occurs spontaneously in these cells^{47,48}.

These contrary actions could be explained by postulating that cells possess a single genetic control element that dictates whether to self renew or to generate committed progeny.

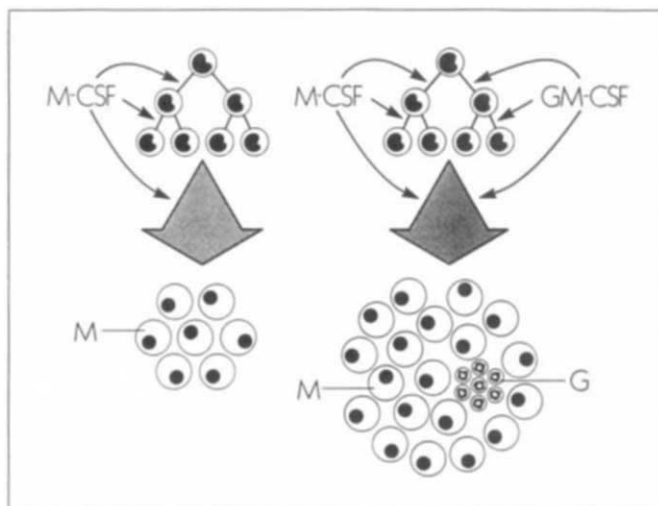


FIG. 3 Bipotential granulocyte-macrophage progenitors *in vitro* can be committed irreversibly to macrophage formation if the initial cell divisions are stimulated by M-CSF. If a combination of M-CSF and GM-CSF is used, there is both enhancement of cell proliferation and competition for differentiation commitment, often resulting in the development of a tight subclone of granulocytic cells (G) within a macrophage (M) colony.

Leukaemia inhibitory factor, or a product generated by its binding to its receptor, could interact with this control element. The actual outcome of this interaction would be determined by the genetic programme of the responding cell. Unfortunately this simple scheme cannot account for the observations that the concentration and nature of the CSF used can determine the lineage entered by the cell during differentiation commitment. It seems likely that some component of the CSF-initiated cascade is concentration-dependent or is unique to each CSF and this is able to determine irreversibly which differentiation lineage is selected in a responding cell that has chosen to become committed.

Induction of maturation

Haemopoietic cells display dramatic morphological changes during the maturation which accompanies and follows the final cell divisions (Fig. 1 and Box). Evidence that the CSFs have a direct influence on maturation is now accumulating, but it has been difficult to obtain because the CSFs are required for the survival of progenitor cells and their maturing progeny *in vitro* and because the CSFs simultaneously stimulate cell division in immature cells. Hence, it could be argued that maturation might simply occur passively after a certain number of cell divisions rather than being directly induced by the CSFs.

One successful approach is the use of a continuous haemopoietic cell line that is responsive to more than one CSF. Cells of the line 32D clone 13 initially proliferate in response to stimulation by both multi-CSF and G-CSF, but differentiate to mature granulocytes only when stimulated by G-CSF. Using low concentrations of multi-CSF, healthy proliferating cells can be observed in the presence or absence of G-CSF; the results indicate that G-CSF does actively induce granulocyte maturation in these cells⁴⁹.

It remains unlikely that the CSFs regulate directly every detail of a maturation sequence. For example, multi-CSF can stimulate the formation of maturing erythroid, granulocyte-macrophage, eosinophilic, megakaryocytic and mast cells (Table 1); it is improbable that it directly regulates all of the many events required to produce each lineage. It is difficult to see how the development of features inappropriate to a particular lineage, such as the formation of eosinophil granules in a maturing macrophage, could be avoided. More probably, a CSF induces a signal that merely initiates a self-sustaining sequential genetic programme of transcriptional activations involved in the matu-

ration of cells in a particular lineage. CSF stimulation induces the synthesis of many cellular proteins, one or more of which may be able to bind to relevant genetic regulatory elements to activate maturation^{50,51}.

The proposed involvement of the CSFs in induction of maturation is similar in principle to part of their role in differentiation commitment: they initiate signalling but the sequence and nature of the events that follow are determined by the cell's own programming.

Prospects for work *in vivo*

I have deliberately concentrated on certain highly simplified *in vitro* models to establish some of the basic features of the control of haemopoietic cell division, commitment and maturation. Obviously the situation *in vivo* is much more complex because the CSFs can interact with other regulators; there may be combined or sequential actions of multiple haemopoietic growth factors, and the possibility of regulator networks in which one factor induces the production of others, all of which have been observed *in vitro*. The supporting, or stromal, cells in haemopoietic tissue are also known to have a strong influence in controlling the self renewal of stem cells and differentiation commitment, no doubt partly because they produce many of the haemopoietic factors, but possibly also through cell-contact-mediated events.

In spite of these potential complexities it is intriguing to observe that the injection of recombinant CSFs into animals⁵²⁻⁵⁶ or patients⁵⁷⁻⁶² closely reproduces the key actions of the CSFs seen in simplified *in vitro* cultures. *In vivo*, as *in vitro*, the CSFs clearly have a capacity to stimulate selectively the production of maturing granulocytes or monocytes; the range of lineages stimulated is expanded as dosages are increased, and stimulation of the functional activity of mature cells is also observed. Whether differentiation commitment is regulated *in vivo* as *in vitro* remains to be established.

The successful introduction of these powerful agents into clinical practice validates the initial use of oversimplified *in vitro* models for investigating basic biological processes, but the caution remains that some of the minutiae observable in culture may not be of much real relevance when manipulating haemopoiesis *in vivo*. □

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ACKNOWLEDGEMENTS. The work from the author's laboratory was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria, The National Health and Medical Research Council, Canberra and The National Institutes of Health, Bethesda.

Alternative packing arrangements in the hydrophobic core of λ repressor

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The random alteration of hydrophobic core positions in the N-terminal domain of λ -repressor, both individually and in combination, shows that there are many ways of repacking the core of the protein. Although the number of functional sequences is limited by constraints on composition, volume and steric interactions, the simple requirement that these positions remain hydrophobic is the main determinant of whether a core sequence is compatible with the wild-type fold.

THE amino-acid residues that interact to form the hydrophobic core of a protein clearly play a key role in determining the protein's structure. Although many amino-acid substitutions can be made at surface positions with no adverse effects, substitutions at internal positions tend to be severely destabilizing¹⁻³. Similarly, within a family of homologous proteins, the most conserved residues, apart from functional amino acids, are those that are buried⁴. Several factors might contribute to the importance of the core. First, the core is composed of hydrophobic

side chains, the shielding of which from solvent (the hydrophobic effect) is thought to be one of the main sources of stabilization energy^{5,6}. Second, crystallographic studies have shown that core side-chains are very closely packed, occupying almost all available interior space with no steric overlaps^{7,8}. A particular protein structure might therefore require a specific set of core residues which can maintain each of these properties. Ponder and Richards⁹ have suggested that by focusing on core residues, it may be possible to predict the structure that a protein sequence will adopt. The success of such predictive schemes, however, will depend on our understanding of the different types of information encoded in a core sequence.

To approach this problem, we have used the technique of cassette mutagenesis to alter randomly sets of residues within the core of a protein of known structure, the N-terminal domain of phage λ repressor^{10,11}. By selecting the resulting mutants for repressor function, we have identified different sequences that specify the λ repressor fold. Our comparison of these sequences highlights what properties of the residues are informationally relevant. By lowering the level of activity required to pass the selection, we were also able to isolate proteins that fold but because of instability or perturbations in the final structure do not function as well as the wild-type protein. The sequence

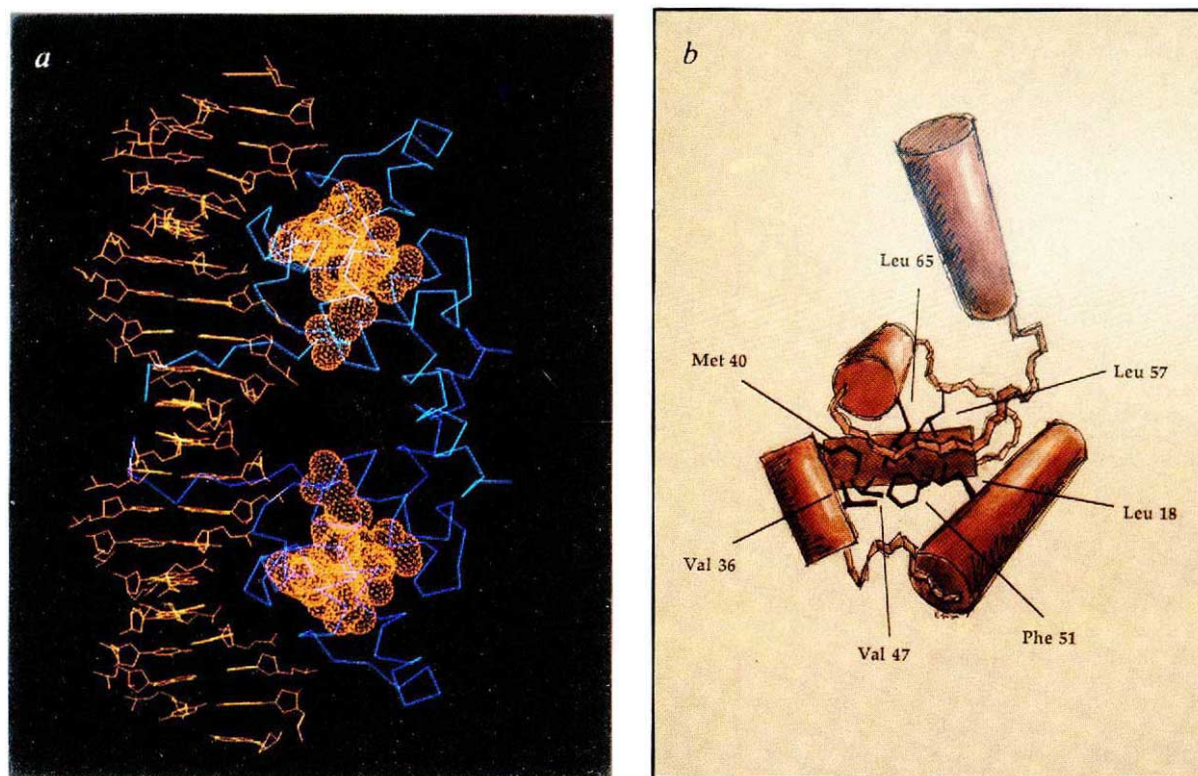


FIG. 1 The hydrophobic core of the N-terminal domain of λ repressor. *a*, The core residues 18, 36, 40, 47, 51, 57, and 65, shown within the complex of the repressor dimer and operator DNA¹¹. The backbone of the dimer is shown in blue whereas the van der Waals surfaces of the core residues are shown in orange. *b*, A sketch of the core residues in one monomer with

the complex rotated 90° about the axis of the DNA such that the DNA would be behind the plane of the page. The side-chains of these residues form a compact unit with a packing density of 0.71 (calculated using the method of Richards²⁶). Each of these side chains has a solvent accessibility of less than 5% of the same side chain, X, in the reference tripeptide Ala-X-Ala^{27,28}.

properties that are conserved in this group of less active proteins, can be used to determine what core information is most basic and essential to a protein's fold. Much about the determinants of protein folds has been learned from similar analyses of sequence variation in families of homologous proteins¹²⁻¹⁶, and our study is essentially an extension of this approach. The main point of difference, however, is that here we focus on the core residues of a set of otherwise identical proteins.

Experimental strategy

Seven buried residues, Leu 18, Val 36, Met 40, Val 47, Phe 51, Leu 57, and Leu 65, which pack against one another in the centre of the globular protein, were deemed to constitute the hydrophobic core (Fig. 1). Mutagenesis was performed (see Fig. 2) and active mutants were selected for resistance to killing by λ KH54, a derivative of phage lambda which lacks a functional repressor gene. Under these conditions the survival of a mutant depends on the expression of an active repressor protein. Furthermore, as the gene encoding the N-terminal domain was under the control of an inducible *tac* promoter, it was expressed at low and high levels, when the cells were grown in the absence

and presence of inducer, respectively. This allows active mutants to be further divided into two phenotypic classes, functional and partially functional. Whereas the former, like wild-type, confer phage resistance when expressed at the uninduced level, the latter confer resistance only when expressed at the induced level. To isolate an additional class of non-functional mutants, those not subjected to the selection were also screened.

Operator binding by the dimeric N-terminal domain requires an extensive set of three dimensional contacts, both between the protein and DNA and between the two protein monomers¹¹. Thus, mutant proteins that retain some degree of operator binding activity must also retain the basic architecture of λ repressor. Because a functional phenotype requires at least 10% wild-type activity *in vivo*³, proteins in this class presumably have structures and stabilities similar to those of the wild-type protein. Mutants in the partially functional class, however, may have structures that are slightly perturbed or destabilized. Nevertheless, these proteins must retain the basic tertiary fold of wild-type λ repressor and, consequently, those properties of the core that are most basic in determining the protein's fold.

In separate experiments, five of the seven core positions were

FIG. 2 The sequences of core mutants obtained in the N-terminal domain of λ -repressor. Residues which differ from the wild-type amino-acid are indicated in bold face. Also given are the estimated core van der Waals volumes in $\text{\AA}^3$ and the effective free energies of transfer from octanol to water in kcal mol^{-1} calculated by summing the individual values for all seven core side-chains ($\Sigma\Delta G_i$). The values for side chain volumes are from Richards²⁶ and the values for free energies of transfer are from Fauchere and Pliska¹⁸. Functional mutants confer resistance to phage λ KH54 under non-inducing conditions, and partially functional mutants confer resistance to phage λ KH54 only under inducing conditions (0.1 mM isopropyl-1-thio- β -D-galactoside). Non-functional 'hydrophobic' mutants do not confer resistance to λ KH54 but have cores composed only of amino acids which have favourable or neutral free energies of transfer. Many additional nonfunctional mutants with one or more hydrophilic residues in the core were isolated but are not shown here. The approximate properties of the partially functional class can be inferred from the properties of two core mutant proteins that have been purified²⁴. The wild-type protein has a melting temperature (T_m) of 54 °C. Two partially functional mutant proteins, Leu $\rightarrow$ Pro 57 and Leu $\rightarrow$ Cys 57, have T_m s of 39 °C and 35 °C, respectively. These changes correspond to reductions in stabilization free energy relative to wild-type ($\Delta\Delta G$) of 2.6 and 3.3 kcal mol^{-1} , respectively. When folded, these proteins have circular dichroism spectra identical to that of wild-type, indicating that each can assume a similar α -helical structure.

METHODS. Mutagenesis was performed by ligating double stranded mutagenic cassettes into a plasmid-borne gene of residues 1-102 of λ repressor. Cassettes were prepared using a variation on the method of Oliphant *et al.*²⁹, modified to span the wide distances required by combinatorial mutagenesis of sequentially non-adjacent positions. Two different oligonucleotides with the appropriate restriction sequences near their 5' ends and nine complementary bases at their 3' ends, were annealed and used to prime each other's second strand synthesis by DNA Polymerase I

Functional																			
18	36	40	47	51	57	65	Vol	$\Sigma\Delta G_i$		18	36	40	47	51	57	65	Vol	$\Sigma\Delta G_i$	
L	V	M	V	F	L	L	549	14.4	L	L	M	V	F	L	L	L	567	15.1	L
L	I	M	V	F	L	L	567	15.2	L	T	M	V	F	L	L	L	537	13.1	L
L	V	L	V	F	L	L	549	15.0	L	V	V	V	F	L	L	L	531	14.4	L
L	V	M	I	F	L	L	567	15.2	L	V	A	V	F	L	L	L	493	13.1	L
L	V	M	V	L	L	L	538	14.3	L	V	M	T	F	L	L	L	537	13.1	L
L	V	M	V	M	L	L	538	13.6	L	V	M	V	I	L	L	L	538	14.4	L
L	V	M	V	F	I	L	549	14.5	L	V	M	V	C	L	L	L	501	13.3	L
L	V	M	V	F	M	L	549	13.8	L	V	M	V	F	P	L	L	521	13.1	L
L	V	M	V	F	L	F	560	14.5	L	V	M	V	F	V	L	L	531	13.7	L
L	V	M	V	F	L	M	549	13.8	L	V	M	V	F	C	L	L	512	13.4	L
L	V	M	V	F	L	V	531	13.7	L	V	M	V	F	F	L	L	560	14.5	L
L	I	M	V	L	L	L	556	15.1	L	V	M	V	F	L	A	L	493	12.5	L
L	M	V	I	L	L	L	556	14.4	L	V	M	V	F	L	C	L	512	13.4	L
L	V	L	I	F	L	L	567	15.8	L	V	M	V	F	L	S	L	499	12.0	L
L	L	M	I	F	L	L	585	15.9	A	V	M	V	F	M	L	L	493	11.9	L
L	V	M	L	L	L	L	556	14.9	L	A	M	I	F	L	L	L	529	14.0	L
L	V	M	T	L	L	L	526	13.0	L	A	M	L	F	L	L	L	529	13.8	L
A	L	M	V	L	L	L	500	13.0	L	C	L	V	F	L	L	L	530	14.7	L
I	L	M	V	L	L	L	556	15.1	L	I	M	T	F	L	L	L	555	13.9	L
L	A	M	L	L	L	L	518	13.7	L	I	V	V	F	L	L	L	549	15.2	L
L	I	C	I	F	L	L	548	15.7	L	L	I	V	F	L	L	L	567	15.8	L
L	I	L	I	F	L	L	585	16.6	L	L	L	V	F	L	L	L	567	15.7	L
L	L	M	I	L	L	L	574	15.7	L	L	V	V	F	L	L	L	549	15.0	L
L	L	M	L	L	L	L	574	15.6	L	M	M	I	F	L	L	L	585	15.2	L
L	M	M	I	M	L	L	574	14.5	L	M	M	L	F	L	L	L	585	15.1	L
L	T	M	L	L	L	L	544	13.6	L	T	L	V	F	L	L	L	537	13.7	L
V	V	M	I	L	L	L	556	14.4	L	T	M	I	F	L	L	L	555	13.9	L
V	V	M	L	L	L	L	538	14.3	L	V	A	I	F	L	L	L	511	13.9	L
I	L	M	L	L	L	L	574	15.7	L	V	C	I	F	L	L	L	530	14.9	L
V	T	M	I	L	L	L	526	13.1	L	V	C	M	F	L	L	L	530	14.1	L
V	M	M	I	L	L	L	538	14.4	L	V	I	C	F	L	L	L	530	14.9	L
									L	V	I	I	F	L	L	L	567	16.0	L
									L	V	L	A	F	L	L	L	511	13.8	L
									L	V	L	T	F	L	L	L	537	13.7	L
									L	V	V	I	F	L	L	L	549	15.2	L
									V	V	M	V	F	F	L	L	542	13.9	L
									V	V	M	V	F	I	L	L	531	13.9	L
									V	V	M	V	F	L	F	L	542	13.9	L
									A	V	M	V	F	M	I	L	493	12.0	L
									L	A	M	F	L	L	L	L	529	13.8	L
									L	C	L	I	F	L	L	L	548	15.5	L
									L	C	M	M	L	L	L	L	537	14.0	L
									L	C	M	F	V	L	L	L	530	14.1	L
									L	I	A	I	F	L	L	L	520	14.7	L

Non-functional Hydrophobic

18	36	40	47	51	57	65	Vol	$\Sigma\Delta G_i$
F	V	M	V	F	L	L	560	14.5
P	V	M	V	F	L	L	521	13.1
S	V	M	V	F	L	L	499	12.0
W	V	M	V	F	L	L	588	15.2
G	V	M	V	F	L	L	473	12.1
L	G	M	V	F	L	L	491	12.7
L	P	M	V	F	L	L	539	13.7
L	W	M	V	F	L	L	606	15.8
L	S	M	V	F	L	L	517	12.7
L	V	M	V	A	L	L	482	12.4
L	V	M	V	S	L	L	488	11.9
L	V	M	V	T	L	L	508	12.3
L	V	M	V	F	A	L	493	12.5
L	V	M	V	F	G	L	473	12.1
L	V	M	V	F	S	L	499	12.0
L	V	M	V	F	Y	L	567	13.4
L	V	M	V	F	L	G	473	12.1
L	V	M	V	F	L	P	521	13.1
L	V	M	V	F	L	W	588	15.2
L	V	M	V	F	L	Y	567	13.4
G	V	M	V	F	V	L	455	11.4
L	P	M	S	F	L	L	507	12.0
A	V	M	F	I	L	L	511	13.3
C	A	M	F	L	L	L	492	12.8
V	A	M	L	I	L	L	500	13.2
V	T	M	I	T	L	L	496	11.1

Klenow fragment. This yielded double stranded molecules approximately twice the length of the individual oligonucleotides. After digestion at the terminal restriction sites, these cassettes were gel-purified, ligated into the plasmid backbone, and the ligated products transformed into *E. coli*. Random alteration of specific codons was achieved by synthesizing the cassette oligonucleotides with an equal mixture of all four bases at the first two codon positions and an equal mixture of G and C at the third position. This procedure generates 32 codons encoding all 20 naturally occurring amino acids³.

TABLE 1

position	wt	Active substitutions				Inactive substitutions	
		single	Functional multiple	single	Partially Functional* multiple	single	
18	L	—	AVI	ACVIM	ACVIM	FWPGSNQ	
36	V	I	ATLIM	ACTLI	ACTLIM	WPGSNHDEKR	
40	M	L	CL	AVL	ACVLI	†	
47	V	I	TLI	TI	ACTLIMF	†	
51	F	LM	LIM	CVIM	CVLIM	ASTDR	
57	L	IM	—	CPVIMF	IMF	AYGSDR	
65	L	VMF	—	ASCTVMF	IF	YWPGNHER	

Summary of acceptable and unacceptable amino-acid substitutions at each core position. Single substitutions are those that occur within the context of wild-type amino acids at all other positions. Multiple substitutions are those that occur simultaneously with substitutions at one or more other core positions.

* Here partially functional substitutions include all active substitutions, both partially functional and functional.

† Inactive substitutions were not isolated at positions 40 and 47 as these positions were not altered individually.

altered individually. Only one to three amino-acid substitutions at each position yield a fully functional protein (Table 2) as is common for buried positions³. In three experiments, the following sets of core residues were then altered randomly: Val 36, Met 40 and Val 47; Leu 18, Val 36, Val 47 and Phe 51; and Leu 18, Leu 57 and Leu 65. In the first experiment 266 out of 20,000 mutants survived the genetic selection under inducing conditions. In the second and third experiments 92 out of 40,000, and 36 out of 15,000 survived, respectively. Of these survivors, 30–100 from each experiment were sequenced and classified as functional or partially functional. The core sequences of the resulting mutants are shown in Fig. 2. Altogether, 31 different functional amino-acid sequences and 62 different partially functional amino-acid sequences were isolated.

Physical constraints

Composition. The cores of all proteins that retain some activity are composed almost entirely of the eight amino acids Ala, Cys, Thr, Val, Ile, Leu, Met, and Phe (Table 2). Most of these residues are apolar and have favourable free energies of transfer from water to organic solvents^{17,18}. This result is consistent with the idea that one of the main energetic contributions to protein stability is the shielding of hydrophobic groups from solvent^{5,6}. However, Thr and, rarely, Ser are allowed within the core in spite of the fact that these hydroxyl-containing amino acids have free energies of transfer that are approximately zero. Amphiphilic side chains almost always satisfy their hydrogen-bonding potential, either intramolecularly or by extending out of the core into solvent¹⁹. The acceptability of Thr and Ser is probably explained by the fact that these side-chains are commonly observed to form a hydrogen bond back to the main chain^{4,20,21}. Conversely, it seems that the amino acids Tyr and Trp, which have highly favourable free energies of transfer but contain relatively polar groups are not acceptable at any of the seven core positions, including positions 57 and 65, where Phe is allowed. These large side-chains may not occur at these totally buried positions in functional proteins because they cannot form the appropriate hydrogen bonds. Combinations in which Trp is acceptable might also be rare because of its large size and unusual shape. It also seems that other polar amino-acid substitutions are unacceptable in the core even in pairs with a hydrogen bonding or salt-bridge partner. This indicates that appropriate geometries into which such substitutions could be placed are rarely found.

Volume. The distribution of the acceptable core van der Waals volumes for both classes of active mutants is centred around the wild-type value, but has a range from ~40 Å³ larger than that of the wild-type to ~60 Å³ smaller than that of the wild-type (Fig. 3). This range corresponds to between +2 to -3 methylene groups. The r.m.s. deviation is 20 Å³ for the functional class and 25 Å³ for the partially functional class. This variation

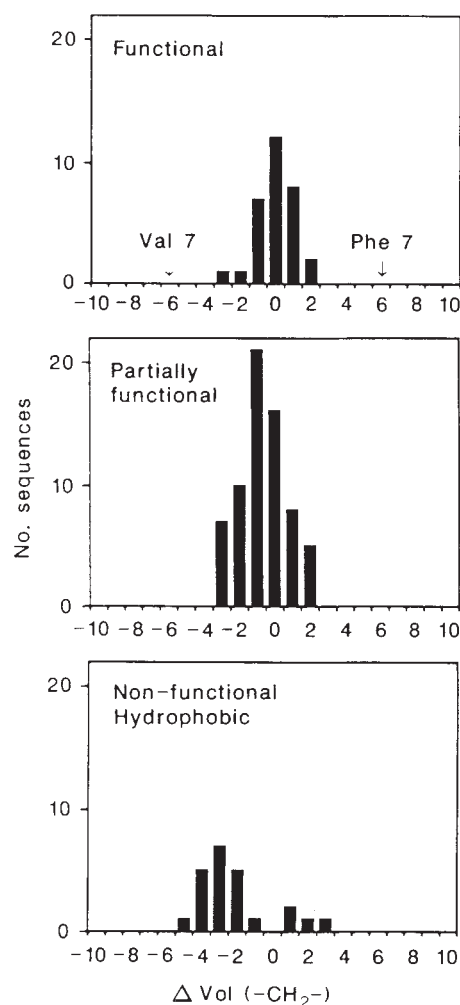


FIG. 3 Distribution of core van der Waals' volumes for all core mutants listed in Fig. 2. Volumes are the sum of the volumes of all seven core side chains and are expressed as the difference from the wild-type volume, in units of methylene groups (18 Å³). The positions at which cores composed totally of phenylalanine or totally of valine would fall are indicated. A core composed totally of Ala would fall at -20 methylene groups. The wild type core volume is 549 Å³. The functional mutants have a mean core volume of 552 Å³ and a standard deviation of 19.6 Å³. The partially functional mutants have a mean core volume of 538 Å³ and a standard deviation of 24.7 Å³. The non-functional hydrophobic mutants have a mean core volume of 513 Å³ and a standard deviation of 38.3 Å³.

suggests that either the protein can tolerate core packing densities from as low as 0.67 to as high as 0.75 or that adjustments in the structure occur, allowing a reasonably constant packing density to be maintained. A similar level of volume variation has been observed in the globin family, where the total volume of the core remains roughly constant with an r.m.s. deviation of 15 Å³ (Ref. 12).

Steric interactions. The observed composition and volume constraints do not account for all the apparent restrictions on acceptable core sequences. Several mutants satisfying both these requirements are non-functional (Fig. 2). We propose that most of the additional restrictions result from stereochemical constraints. The specific arrangement of atomic volume must be important because the core side-chains must pack together avoiding steric clashes within the limits of the active protein structure. Steric interactions probably also account for the distinction between functional and partially functional mutants, as only certain side-chain combinations are likely to fit together efficiently. Indeed, many mutants with the same core volume and composition as mutants within the functional class are only partially functional. For example, the mutant Val 36/Leu 40/Val 47 is fully functional whereas the compositionally identical mutant Leu 36/Val 40/Val 47 is only partially functional. Clearly, removing a β -branched side-chain from position 36 and adding it to position 40 must cause some distortion in structure and/or reduction in stability. The delineation of limits for the steric constraint, however, is far more difficult than for the other constraints. Volume and composition are intrinsic properties of a core sequence, whereas steric compatibility is dependent not only on the set of core residues but also on the protein backbone position and the entire protein structure. It is unclear how these factors might vary for each particular mutant. For these reasons, an attempt to define the steric constraint is beyond the scope of the present study. Clear answers will require detailed structural analyses of the mutant proteins.

Informational importance of constraints

Three physical constraints on active core sequences have been clearly identified. Yet even if a particular constraint is physically important, it may not have a high degree of informational importance. For example, a certain feature of a sequence may be absolutely essential for an active protein. If this feature, however, is maintained in 90% of all possible sequences, active and inactive, its informational significance is reduced, as identifying the feature will not help to discriminate between most active and inactive sequences. It is the informational content of a sequence that one must focus on to understand its structural meaning.

How important are the composition, volume, and steric constraints in determining whether a particular core sequence will fold into λ repressor? The informational importance of each

constraint can be roughly assessed by calculating the reduction in the number of allowed sequences that results from the application of these constraints. This is straightforward for the compositional and volume constraints, but difficult for the steric constraint. Nevertheless, we know the approximate fraction of sequence space that is acceptable and can therefore estimate the total number of sequences passing all three constraints. This, in turn, allows us to determine the fraction of compositionally and volumetrically allowed sequences which also satisfy the steric constraint. How these constraints combine to define the allowed sequence space is depicted conceptually in Fig. 4.

Of the 8,000 (20^3) possible sequences resulting from the random alteration of Val 36, Met 40 and Val 47, 512 (8^3) are composed only of the eight allowed hydrophobic amino acids. Thus, 6% of the total sequence space is allowed by the hydrophobic constraint. When the core van der Waals volumes of all possible sequences are calculated, 5,848 sequences are found to fall within the limit of -3 to $+2$ methylene units of that of the wild-type protein. Thus 73% of total sequence space is allowed by the volume constraint. When both the hydrophobic and volume constraints are applied together, 425 sequences, or 5% of the total, are acceptable. Our data indicate that approximately 110 amino-acid sequences, or 1.4% of the possible sequences, are active, of which 20, or 0.3%, are fully functional. Similar analyses for the other random mutagenesis experiments are shown in Table 2.

These calculations for the three combinatorial random mutagenesis experiments indicate that hydrophobicity and stereochemistry are the most important types of information as they impose the largest reductions in the number of possible sequences that can fold into a specific structure or family of homologous structures. Although the volume constraint is clearly of physical importance, it is numerically the least restrictive because most sequences fall within an acceptable range. For example, in the case discussed above, it is obvious that the core sequences Phe-Phe-Phe or Ala-Ala-Ala will not be volumetrically acceptable. Because 73% of all random tripeptides are acceptable, however, the volume constraint is not an effective means of discriminating between most active and inactive sequences.

Conclusions

The survey presented here has allowed us to look at general rules and patterns concerning packing of the hydrophobic core. The broad nature of this problem requires the examination of many sequences, which in turn, necessitates the use of a low resolution approach to structure and stability. We have therefore divided mutants into two qualitative classes on the basis of activity *in vivo*. Our aim is not to assign free-energy values to particular core interactions nor to probe detailed alterations in the protein's structure and properties^{22,23}. To address these

TABLE 2 Reduction in allowed core sequences imposed by constraints

Applied constraints	Experiment	Number of core amino-acid sequences passing constraints		
		I (V36 M40 V47)	II (L18 V36 V47 F51)	III (L18 L57 L65)
None		8,000 (100%)	160,000 (100%)	8,000 (100%)
Volume		5,848 (73%)	100,763 (63%)	5,052 (63%)
Composition		512 (6%)	4,096 (2.5%)	512 (6%)
Volume and composition		425 (5%)	2,846 (2%)	335 (4%)
Volume, composition, and steric:				
active sequences		~110 (1.4%)	~690 (0.4%)	~25 (0.3%)
fully functional sequences		~20 (0.3%)	~270 (0.2%)	~6 (0.1%)

For an experiment in which n positions are randomly altered, the total number of possible sequences is 20^n . The total number of compositionally acceptable sequences is 8^n , as only Ala, Cys, Thr, Val, Ile, Leu, Met, and Phe were found to be acceptable (Pro and Ser which were each found at only one position in one mutant are excluded). The number of sequences allowed by the volume constraint is the number of possible sequences which have core volumes within -3 to $+2$ methylene units of the wild-type volume. The number of sequences passing all three constraints is estimated from the fraction of mutants surviving the selection. A definite lower limit for these estimates is given by the total number of active sequences actually isolated and sequenced. These values are: experiment I, 42 active, 8 fully functional; experiment II, 54 active, 22 fully functional; experiment III, 24 active, 6 fully functional.

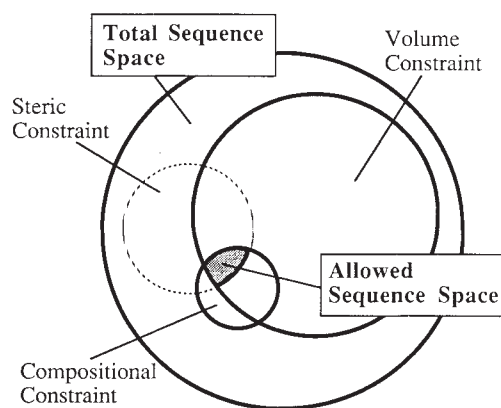


FIG. 4 Venn diagram depicting schematically the relative importance of the volume, composition, and steric constraints. The smaller the fraction of total space enclosed by a constraint, the greater its information content. The limits of the steric constraint have not been clearly delineated and are thus depicted with a dotted line. The limits of the steric constraint within the context of the other constraints, however, can be estimated from the total number of allowed sequences.

higher resolution problems, biochemical and crystallographic analyses of the mutants will need to be performed.

Relevant core sequence information. By examining the properties of the fully functional mutants, one can focus on what specifies the detailed structure and stability of a protein. From these experiments, it seems that only a limited set of core sequences possess all of the information required to specify fully functional λ repressor. All of these sequences carry important compositional and steric information. The hydrophobic constraint imposes a 20 to 50-fold reduction in the number of allowed sequences, and the sequentially applied steric constraint imposes a further 10 to 40-fold reduction. The great importance of steric compatibility at the functional level is perhaps most clearly illustrated by the observation that the number of functional substitutions allowed at a particular position is dependent on the number of other core mutations occurring simultaneously (Table 1). The spectrum of functional substitutions at positions 18, 36, 40, 47, and 51 increases when multiple core mutations are allowed, whereas the spectrum at positions 57 and 65 decreases. The core is clearly an interdependent unit; what substitutions are allowed at a position is dependent on the context. Only a limited set of core residues are able to pack together efficiently into a fully active protein. These results suggest that the concept of tertiary templates will prove useful in delineating such limited sets of well-packed, stable structures⁹.

The interdependence of the acceptability of core substitutions has additional implications for the evolution of proteins. During evolution proteins presumably face selections akin to our functional level of activity. Unlike our experiments, however, in which interacting positions are allowed to vary in concert, evolutionary change probably occurs through a series of single mutations. From our results, it seems that certain single mutations in the core are unfavourable unless they occur simultaneously with complementary mutations at adjacent core positions. In this way, as a consequence of the close packed nature of the core, the pathway of protein evolution is probably highly directed and limited to certain regions of sequence space¹⁴. Although an alternative packing arrangement in a protein may be fully active, it may remain unexplored because the protein would have to pass through a series of inviable evolutionary intermediates to get there.

Essential core sequence information. What core-encoded information is required to specify the basic fold of a protein, without

regard to its detailed structure or stability? We have addressed this question by relaxing our functional requirement and isolating the partially functional mutants. The resulting increase in acceptable sequences reflects a 3 to 5-fold decrease in the reduction imposed by the steric constraint. At this level of activity there is no clear dependence of positional variability on the number of other mutations, unlike the functional class (Table 2). The steric constraint therefore seems to play an important part in specifying the detailed properties of a protein rather than in determining its basic architecture. The most essential informational property of the core is simply that it must remain hydrophobic. By extrapolation, it is conceivable that if the level of selection were lowered further, many of the other mutant proteins that satisfy the hydrophobic requirement but which are classified here as non-functional might also survive. These proteins may fold into a structure whose activity is just below the level detectable by our partially functional selection. This seems to be the case for the non-functional protein Leu $\rightarrow$ Ala 57, which has been purified and biochemically characterized²⁴. Although this protein has a melting temperature (T_m) of 20 °C, whereas the wild-type protein has a T_m of 54 °C, it seems, according to circular dichroism measurements, to fold into an α -helical native-like structure at low temperatures. Thus, such mutants maintain a unique free-energy minimum, albeit a shallow one, near to that of the wild-type protein.

Our results suggest that hydrophobicity is the most essential feature of a core sequence. This does not mean that the other properties of core residues are physically or energetically unimportant, but rather that at this level they are fairly degenerate and provide little additional information, given that the range of natural hydrophobic amino-acid sizes and shapes is small relative to the range of allowed variability. Clearly, not all hydrophobic core sequences can fold, but because such a large fraction of hydrophobic combinations are active, the most effective means of discriminating between active and inactive core sequences may simply be to search for those sequences that satisfy the hydrophobic composition constraint.

How might these findings be applied to the broader question of understanding the structural meaning of an entire protein sequence? In general, the identity of individual surface residues is unimportant—most information is encoded in the core residues. We have shown, in the case of λ repressor, that most of the basic non-degenerate information in the core is simply the core's hydrophobic composition. If these findings can be generalized to other globular proteins, then perhaps most of the information determining whether a sequence has the potential to adopt a particular basic fold lies within the simple pattern of hydrophobic residues. Implicitly, the pattern of hydrophilic residues in the sequence must also be important, as an excess of hydrophobic residues at surface positions would present competing hydrophobicity patterns. Lau and Dill²⁵ have shown that even such a simple pattern contains much structural information. Chains with only two components, hydrophobic and hydrophilic residues, have been modeled on a two dimensional square lattice. An exhaustive search of possible conformations reveals that only a small set of basic chain topologies permit the maximal burial of hydrophobic residues in a compact core and the simultaneous exposure of hydrophilic residues. Therefore, algorithms that try to identify the basic topologies consistent with such a simplified pattern may prove to be useful first-order approaches to *ab initio* structural prediction. Structures generated by such procedures might then be refined and evaluated by considering more detailed properties, including steric interactions. Template methods based largely on the patterns of hydrophobic residues have also proven useful in identifying proteins of a particular structural class^{15,16}. Although such an abstract representation of a polypeptide chain obviously ignores many important properties of a sequence, it may be sufficiently accurate to be an effective means of simplifying the complex problem of structural prediction. □

Received 6 December 1988; accepted 4 April 1989.

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ACKNOWLEDGEMENTS. We thank D. Parsell, who isolated and studied several of the mutants discussed here, J. Bowie, R. Davenport, J. Hu, P. Kim, P. Lansbury, C. Pabo, J. Reidhaar-Olson, L. Regan, and K. Shoemaker for assistance and advice, and J. Ponder and F. Richards for suggestions. This work was supported by the NIH. W.A.L. is a Howard Hughes Medical Institute Doctoral Fellow.

LETTERS TO NATURE

Reappearance of the annihilation line source at the Galactic Centre

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AN intense 511-keV positron annihilation line source was detected from the direction of the Galactic Centre (GC) during the 1970s^{1,2}. Positrons are assumed to be produced by high-energy astrophysical processes at or near the Galactic Centre, and to subsequently annihilate on ambient electrons in the vicinity of the source to generate characteristic 511-keV gamma rays. The source disappeared in the early 1980s and remained in a low state through at least 1984^{3–5}. Here we report the results of new balloon-borne measurements of γ -ray emission from the GC, using a germanium telescope. The 511-keV line was detected at a flux level of $(9.8 \pm 1.9) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$ on 1 May 1988 and $(12.3 \pm 1.6) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$ on 29 October 1988. Apparently the compact object has re-emerged sometime between 1984 and 1988. Flux was also measured from a point in the Galactic Plane (GP) 25° west of the GC, and was found to be $(2.4 \pm 1.7) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$. Diffuse flux from the GP has been reported⁶, but these new observations suggest that the model used in ref. 6 may not be correct.

Previous measurements are summarized in Fig. 1 and reviewed in refs 7 and 8. The Ge instruments, which have a relatively narrow field of view (FOV), located the compact source to within $\sim 8^\circ$ of the GC during the 1970s. Positive satellite detections by the Solar Maximum Mission (SMM), reported when concurrent balloon experiments yielded null results, indicate that there is a diffuse component; this is seen more effectively by the large-FOV SMM NaI detectors^{6,9–11}.

Our Gamma-Ray Imaging Spectrometer (GRIS)^{12,13} consists of an array of seven high-resolution Ge detectors surrounded by ~ 400 kg of NaI in active anticoincidence. A series of entrance apertures in the NaI shield define a FOV of 17° full width at half maximum (FWHM) at 511 keV. The effective area at 511 keV is $\sim 85 \text{ cm}^2$. Observations of the GC direction (right ascension $\alpha = 17^{\text{h}} 42^{\text{m}}$, declination $\delta = -29^\circ 0'$) were made at mean atmospheric depths of 4.8 g cm^{-2} on 1 May and 5.7 g cm^{-2} on 29 October over Alice Springs, Australia. Data were accumulated in alternating 20-min target-background segments, with some background segments taken before and some after the target segments. For background the telescope was maintained at the same zenith angle but rotated in azimuth so as to minimize the extent of the GP in the FOV. Azimuth offset angles varied

from 200° to 240° . Background fields were examined to make certain that no γ -ray sources were contained in them. A total of 11 hours of GC target and background data were accumulated on the first flight, and 10 hours on the second flight, following the same observing program. An additional 7 hours of target and background data were accumulated on 30 October at a mean depth of 4.3 g cm^{-2} from a point in the GP 25° west of the GC ($\alpha = 16^{\text{h}} 22^{\text{m}}$, $\delta = -48^\circ 42'$).

Counts in each detector were accumulated during each segment in channels 0.25 keV wide. These data were energy-calibrated in flight by reference to instrumental lines of known energy and summed over detectors into spectra with a binwidth of 1 keV. After division by segment accumulation time, background spectra were subtracted from target spectra. The differences were then corrected for experiment dead time ($\sim 10\%$) and divided by atmospheric transmission and total effective area, to form flux estimates as shown in Fig. 2. Estimates from different target-background pairs were weighted inversely according to variance and averaged to obtain a final estimate and variance.

GC flux estimates near 511 keV for 29 October were fitted to a single gaussian line plus constant but unequal continua above and below 511 keV, to allow for a Ps-like continuum below the line. (Ps denotes an electron-positron bound state.) The fit was good ($\chi^2 = 6.6$ for 16 degrees of freedom) and confidence intervals for each line parameter, determined by letting

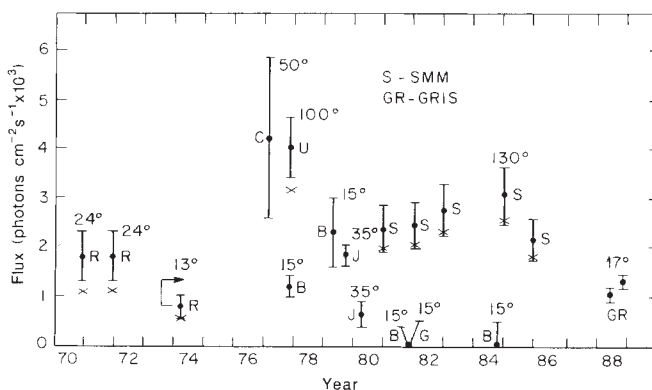


FIG. 1. Observations of 511-keV line emission from the direction of the GC. S, SMM; GR, GRIS; B, Bell/Sandia; C, CESR (France); G, GSFC/CENS (France); J, JPL/HEAO-3; R, Rice Univ.; U, Univ New Hampshire. The field of view (FOV) of each instrument is indicated. The crosses represent line-flux corrections made for an assumed positronium ($\text{Ps} = e^+ + e^-$ bound state) fraction of 0.9. The NaI instruments unavoidably include some three-photon Ps continuum in the line. The 1974 Rice measurement was made in a direction $\sim 5^\circ$ off the GC and needs to be corrected, as indicated, to correspond to a point source at the GC. The B, C, G, J and GR observations were made with high-resolution Ge detectors. A 511-keV line upper limit of 0.8×10^{-3} photons $\text{cm}^{-2} \text{s}^{-1}$ (3σ) has been reported for the bright GC hard-X-ray object 1E1740.7–2942 on 12 April 1988³⁰.

χ^2 increase by unity while being minimized with respect to all remaining parameters, were as follows: line flux = $(12.3 \pm 1.6) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$, line centre = 511.22 ± 0.28 keV, FWHM = 4.0 ± 0.5 keV. Widths of nearby instrumental lines at 439 and 472 keV, formed from target plus background spectra summed over detectors, were 1.8 keV, indicating that the cosmic annihilation line was well resolved with a width of $\sim 3.6 \pm 0.5$ keV, implying a temperature $\leq 10^5$ K for the stopping medium. Attempts to describe the line as the sum of two gaussians¹⁴ did not improve the fit. The cosmic 511-keV line represents a 35% intensity enhancement over an instrumental line whose width is 2.9 ± 0.1 keV.

Attempts to repeat the above analysis for the 511-keV feature observed from the point in the GP were impeded by poor statistics. However, if one constrains the line centre to be at 511 keV, a value of $(2.4 \pm 1.7) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$ is obtained for the measured flux and $5.5 \pm_{3.9}^{4.2}$ keV for the FWHM. Average shield transmission for a point source at the GC during the GP pointing was 2%. Thus, the above value should be reduced by $\sim 10\%$ and divided by our effective aperture, giving a value of $(7 \pm 5) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1} \text{rad}^{-1}$ for the distributed source strength 25° away from the GC. The SMM workers⁶ assumed that the distributed emission comes from regions of current star formation and that its intensity profile is proportional to the CO distribution in the Galaxy¹⁵. As shown in Fig. 3, the new GP result coupled with results obtained in 1981 and 1984 by similar narrow-FOV instruments, when the compact object was not emitting, suggests that this model for the diffuse source may not be correct.

The flight of 1 May was the maiden flight for GRIS, and unanticipated difficulties related to very-high-energy cosmic-ray events degraded the energy resolution to ~ 5 keV at 511 keV and distorted the line shape, thus frustrating attempts to determine the line profile. Nevertheless, the net flux spectrum formed as described above contains a highly significant feature at 511 keV. Continuum net flux values determined in the intervals 464–503 keV and 517–556 keV were interpolated inwards to 511 keV and subtracted from the net flux in the interval 504–516 keV, yielding a line flux estimate of $(9.8 \pm 1.9) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$.

Although no real evidence exists for source variability between the two GRIS GC observations, the combined result, $(11.3 \pm 1.2) \times 10^{-4}$ photons $\text{cm}^{-2} \text{s}^{-1}$, differs from the three combined null results^{3–5} achieved in 1981 and 1984 by similar narrow-FOV Ge instruments at the 4σ level. The probability of this occurring by chance is ~ 1 in 10^4 . Hence we conclude that a compact object has become active after 1984. This conclusion is supported by the GP pointing, which implies the presence of either a point source or a distribution that is highly peaked towards the GC. Additional confidence in our procedures is obtained from our observations of supernova 1987A made during these same

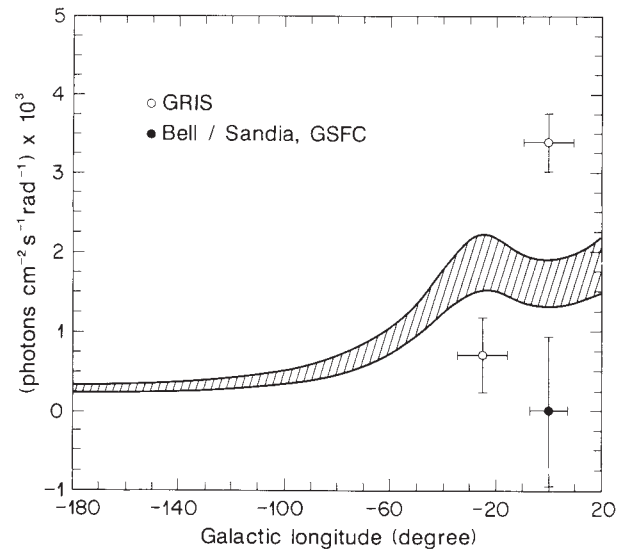


FIG. 3 The GRIS 511-keV flux results, plotted per radian of entrance aperture. Also shown is the diffuse CO-like distribution postulated to explain the SMM observations⁶ between 1981 and 1986. The shaded region is bounded by experimental limits to the best fit of the CO profile to the SMM data⁶. Clearly this particular diffuse distribution alone will not account for the GRIS results. Also shown is the combined result from three balloon flights made with similar Ge instruments in 1981 and 1984.

flights. These data show no net signal at 511 keV. For an isotropic source at a distance of 8 kpc, the line flux requires $\sim 4 \times 10^{42}$ annihilations per second, corresponding to a luminosity of $\sim 7 \times 10^{36}$ erg s^{-1} . If all of these annihilations occur via the bound Ps state then these numbers are four times larger, with the additional luminosity appearing in a Ps continuum.

Considerable evidence exists in the GRIS data for a low-energy 'tail' on the 511-keV line. This tail is now being studied. In the past it has been interpreted as a three-photon Ps continuum. From its intensity relative to the 511-keV line, a large Ps fraction has been inferred¹⁴. However, a similar tail could be generated by Compton scattering from an attenuating medium^{16,17} or by gravitational redshifts near a compact object. The shape and intensity of the tail may allow us to make a choice from these possibilities.

The variability observed about a decade ago inspired many attempts at theoretical modelling. Favoured models^{18–21} invoked black holes with masses between 10^2 and $10^6 M_\odot$, although supernova²² and pulsar²³ models have also been considered. In the low-mass black-hole models, electron-positron pairs are

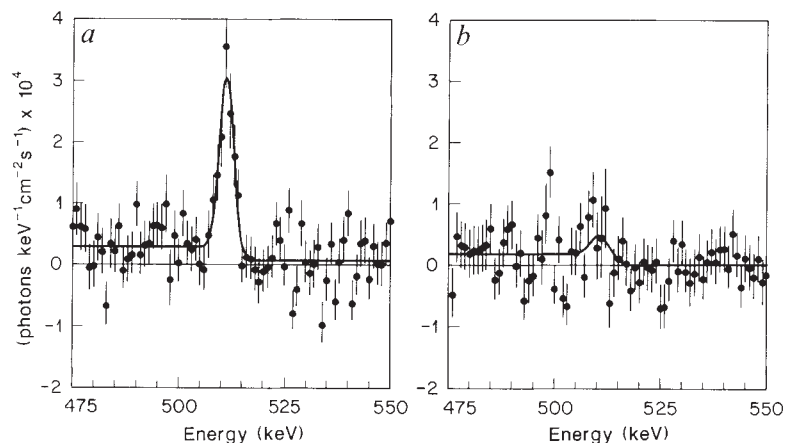


FIG. 2 Background-subtracted spectra obtained from the Galactic Centre (GC) on 29 October (a) and from a point in the Galactic Plane (GP), 25° west of the GC, on 30 October (b). The smooth curves represent best fits from which flux estimates were derived.

produced by γ -ray photon-photon scattering in a hot, dense accretion disk from which some positrons escape to annihilate in surrounding gas clouds. Variability arises from changes in the accretion rate. The high-mass models incorporate rotating black-hole dynamos that electrostatically generate beams of $e^- - e^+$ pairs and γ -rays along the rotation axis. The interaction of the emitted beam with a moving cloud produces the annihilation radiation and provides a mechanism for variability. A high-mass black hole must necessarily be at the dynamical centre of the Galaxy, whereas a low-mass black hole need not.

Recently, a tentative identification of the positron source with the X-ray pulsar GX1+4 was suggested²⁴, based on the similarity of the X-ray and γ -ray light curves over 18 years, the positional agreement of the sources (galactic longitude $l^{\text{II}} = 1.9^\circ$, latitude $b^{\text{II}} = 4.8^\circ$) and the unusual properties of GX1+4. Although no modelling of GX1+4 (a magnetized neutron star with an M6III red-giant companion) as a positron source has yet been published, it is natural to think that a version of the accretion-driven dynamo mechanism proposed²⁵ for some low-mass X-ray binaries may be applicable here. Such a dynamo could electrostatically produce the required number of positrons. The variability might arise either from an eclipse of the positron source²⁴ (the orbital period of GX1+4 is unknown) or from an episodic ejection of mass from the M giant, smothering the X-ray source²⁶. The GRIS data are being searched for the characteristic 2-min pulsation period of such a source.

It is therefore desirable to locate the γ -ray object using imaging and scanning techniques and to frequently monitor the X-ray/ γ -ray flux with the GRO/OSSE²⁷ and GRANAT/SIGMA²⁸ detectors, with balloon instruments such as GRIS, and with the Ge spectrometer on the proposed Nuclear Astrophysics Explorer mission²⁹. $\square$

The unseen companion of HD114762: a probable brown dwarf

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BROWN dwarfs are substellar objects with too little mass to ignite hydrogen in their cores. Despite considerable effort to detect brown dwarfs astrometrically¹⁻⁴, photometrically⁴⁻⁹, and spectroscopically¹⁰⁻¹², only a few good candidates have been discovered. Here we present spectroscopic evidence for a probable brown-dwarf companion to the solar-type star HD114762. This star undergoes periodic variations in radial velocity which we attribute to orbital motion resulting from the presence of an unseen companion. The rather short period of 84 days places the companion in an orbit similar to that of Mercury around the Sun, whereas the rather low velocity amplitude of about 0.6 km s^{-1} implies that the mass of the companion may be as low as 0.011 solar masses, or 11 Jupiter masses. This leads to the suggestion that the companion is probably a brown dwarf, and may even be a giant planet. However, because the inclination of the orbit to the line of sight is unknown, the mass of the companion may be considerably larger than this lower limit.

For more than ten years we have been monitoring carefully the radial velocities of a few dozen stars with the goal of establishing an improved set of velocity standards for the International Astronomical Union¹³. Two different instrument systems have been used: digital speedometers operated by the Center for Astrophysics (CfA) at the Oak Ridge and Whipple observatories¹⁴, and CORAVEL spectrometers at the Haute-Provence and ESO-La Silla observatories¹⁵. One of these standard stars is HD114762 (=BD+18°2700=SAO100458; RA = 13:09:54.53, dec. = +17:46:55.4 [1950], $V = 7.3$). With a $B-V$ colour of 0.53 and surface temperature of about 5,800 K, it closely resembles the Sun, except for a lower abundance of chemical elements heavier than helium, by 0.8 dex (where 0.8 dex = $10^{0.8}$, dex being the interval in powers of 10) (ref. 16). The star has a high proper motion and was therefore included in the survey of Carney and Latham¹⁷, who determine a photometric distance of 28 pc (about 90 light years).

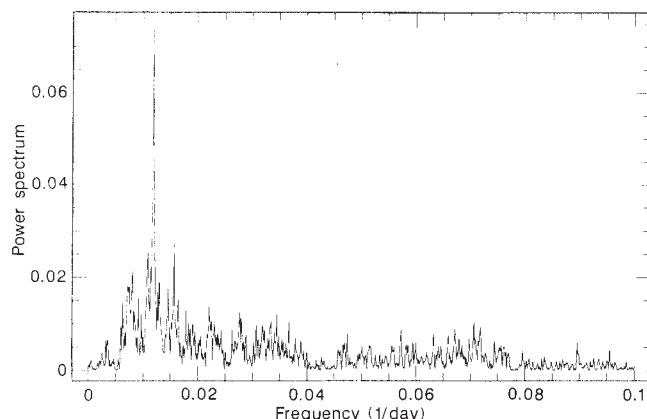


FIG. 1 The power spectrum in relative units for all 280 velocity observations of HD114762 in the combined CfA and CORAVEL data set.

Received 16 February; accepted 5 April 1989.

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ACKNOWLEDGEMENTS. We wish to acknowledge the efforts of the other members of the GRIS team: N. Corlis, S. Derdeyn, C. H. Ehrmann, A. F. Hutters, T. Kovacs, W. Locher, L. Nelson, T. Nolan, R. Smith, D. Sayers, P. Stang and J. Stiles. Launch and recovery support was provided by the crew of NSF under the leadership of R. Kubara.

On 1 April 1988, during test observations with a new fibre feed for the digital speedometer at the Oak Ridge Observatory¹⁸, we noticed a possible variation of HD114762. An examination of the 63 observations taken over the preceding seven years disclosed a periodic variation, with a period of 84 days. The periodicity was subsequently confirmed by the independent CORAVEL data and by extensive additional observations obtained with the CfA speedometers. The power spectrum¹⁹ for the full combined data set of 280 measurements is shown in Fig. 1. The prominent peak dominating Fig. 1 corresponds to a period of 84.02 ± 0.1 days for a pure sine function with half amplitude of about 0.5 km s^{-1} . Even though the error in each individual velocity observation, typically 0.4 km s^{-1} , is similar to the derived amplitude, Fig. 1 leaves no doubt that the data include a periodic modulation.

Are there astrophysical phenomena, besides orbital motion, which might mimic the small periodic velocity modulation found for HD114762? Simple pulsation can be ruled out; given the observed velocity amplitude and period, the star would approximately double its radius by such pulsations. The corresponding light variations would be larger than the upper limit inferred from the available photometry. For example, the fourth edition of the Geneva Catalogue²⁰ lists 24 *V* measurements of HD114762 distributed in phase over the 84-day period, with an r.m.s. amplitude of about 0.006 mag.

Another source of apparent velocity shifts in stellar lines at the level of a few tens of m s^{-1} might be atmospheric circulations or surface activity such as spots combined with stellar rotation. However, such phenomena cannot produce strictly periodic phenomena lasting over many cycles with amplitudes as large as a few hundred m s^{-1} in an old dwarf star²¹. Thus we suggest that the velocity variations seen in HD114762 must be due to orbital motion induced by an unseen companion.

Table 1 lists the parameters of our orbital solutions based on the CfA data alone, CORAVEL data alone, and the full combined data set, derived using our own computer codes with equal weight assigned to each observed velocity. The good agreement indicates that our orbital solutions are robust. Note that no adjustment to the velocity zero points was required when combining the CfA and CORAVEL data. In Fig. 2 we plot the orbital solution for the full combined data set, together with the individual observed velocities.

For a spectroscopic binary with a primary of mass M_s , an unseen companion of mass M_c , and an orbital plane inclined to our line of sight with an angle i , the mass function can be approximated by $(M_c \sin i)^3 / M_s^2$, if M_c is much smaller than

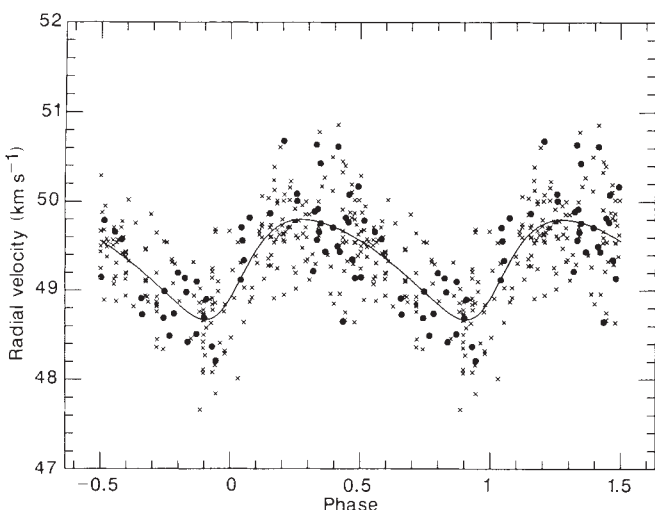


FIG. 2 The orbital solution for the combined data set. The continuous line is the orbital solution with the parameters of Table 1. The CfA velocities are denoted by crosses, the CORAVEL velocities by filled circles.

TABLE 1 Orbital solutions

	CfA	CORAVEL	Combined
Period (days)	84.03 ± 0.14	83.91 ± 0.09	84.05 ± 0.08
System velocity, γ (km s^{-1})	49.31 ± 0.03	49.39 ± 0.06	49.35 ± 0.04
Orbital half-amplitude, K (km s^{-1})	0.55 ± 0.04	0.75 ± 0.12	0.57 ± 0.04
Eccentricity, e	0.26 ± 0.07	0.30 ± 0.15	0.25 ± 0.06
Longitude of periastron, ω ($^\circ$)	237 ± 11	280 ± 16	235 ± 10
Epoch, T (Julian date - 2440000)	$5,029 \pm 5$	$5,033 \pm 4$	$5,027 \pm 4$
Mass function ($10^{-6} M_\odot$)	1.3 ± 0.3	3.1 ± 1.5	1.4 ± 0.3
Number of observations	230	50	280
r.m.s. residuals (km s^{-1})	0.42	0.39	0.42

M_s . For HD114762 this leads to

$$M_c \sin i = 0.011 \pm 0.001 M_\odot$$

where we have used the fact that $M_s \approx M_\odot$. This value of M_c is much smaller than $0.08 M_\odot$, the traditional dividing line between brown dwarfs and stable hydrogen-burning stars²²⁻²⁵. Actually, this value is even smaller than $0.02 M_\odot$, the proposed dividing line between a brown dwarf companion (formed by fragmentation) and a planetary companion (formed by accretion in a cold disk of material around the star)²⁶. Thus the unseen companion of HD114762 is a good candidate to be a brown dwarf or even a giant planet. However, we cannot be certain of the mass of the companion, because we do not know $\sin i$. The probability of an orbit having inclination i is proportional to $\sin i$, if the sample is unbiased and the orbits are orientated randomly. For the companion of HD114762 to be massive enough to burn hydrogen stably, the orbit would have to be within 8° of being viewed face-on, which has a probability of less than 1%.

Finding the orbital inclination of this system is clearly of great importance. In principle this might be done by determining the astrometric orbit, as has been accomplished recently in the case of Gliese 623 (ref. 11). However, the displacement expected for HD114762 is less than 10^{-3} arcsec, too small to be detected in the near future. Another way to pin down the orbital inclination would be to observe eclipses of HD114762 by its unseen companion. Because an eclipse could occur only for an orbit viewed within half a degree of being edge-on, this possibility is rather unlikely. Nevertheless, eclipses should be looked for, as they might allow determinations of the size and temperature of the companion, as well as its mass.

How many low-mass companions are there in the solar neighbourhood? Although we consider answers based on a single example to be unsatisfactory, nevertheless it is illuminating to consider how many other stars have had their velocities scrutinized as carefully as HD114762. The number is of the order of 50, if we consider the stars that have been observed intensively as candidates for new IAU radial-velocity standard stars¹³. HD114762 is the only one of these to have so far shown an unquestionable low-amplitude periodic modulation. However, a few other of these stars also show small velocity variations which may yield convincing orbital solutions in the future. We also note that one of the four well observed giant stars which were chosen to serve as the fundamental standards for the Cambridge radial-velocity spectrometer, HR152, was subsequently shown to have a spectroscopic orbit²⁷. With a period of 576 days and an amplitude of 0.69 km s^{-1} , the mass of the companion would be 35 Jupiter masses for an orbit viewed edge-on. Thus, brown-dwarf companions might not be as rare as suggested by Campbell *et al.*¹⁰ and by Marcy and Benitz¹², based on their studies of 18 and 70 stars respectively. To learn more about the frequency and characteristics of low-mass

companions, we need the results from surveys to monitor the radial velocities of large samples of stars with well-defined selection criteria.

Despite considerable efforts to detect planetary systems around other stars, the Solar System itself is the only certain example that we have. Although the detection of a planet as small as the Earth around another star by spectroscopic techniques will be difficult, the discovery of low-mass companions around other stars by this and other techniques²⁸ is an important step towards understanding the frequency with which planets and planetary systems occur. □

Received 18 January; accepted 15 March 1989.

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ACKNOWLEDGEMENTS. We thank J. Andersen, J. Caruso, R. Davis, R. McCrosky, O. Rodrigues, G. Schwartz, S. Tokarz, G. Torres and J. Zajac for help with the observations and data reduction at CfA, and A. Duquenois, J. L. Halbwachs, M. Imbert, J.-C. Mermilliod and L. Prévot for their contributions to the CORAVEL observations. T.M. thanks A. Bar-Nun for discussions and suggestions. This work was supported by the Smithsonian Institution, the United States-Israel Binational Science Foundation and the Swiss National Foundation for Scientific Research.

Primordial binaries and globular cluster evolution

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MANY of the globular clusters in our Galaxy have probably undergone core collapse, and are currently re-expanding^{1,2}. This re-expansion requires a central energy source. Previously proposed mechanisms are either inefficient or may produce unacceptably bright cores³. Here we explore the most conservative solution to this problem. We suggest that primordial binaries, for which there is now direct evidence^{4–6}, could provide the necessary energy. We show that this mechanism leads to relatively large core sizes, containing ~1% of the total cluster mass. Such a cluster would have a resolvable core (with a size of the order of arcseconds) which would consist mostly of binaries.

Globular clusters are known to undergo core collapse, driven by two-body relaxation which conducts heat from the centre to the outskirts^{7,8}. For a system composed of point masses (without binaries) this process formally produces infinite density in a few half-mass relaxation times t_{rh} (ref. 9). The median value is $t_{\text{rh}} \approx 2 \times 10^9$ yr, which suggests that many globular clusters have already collapsed⁹. Indeed, the observed fraction of clusters with unresolved cores¹ is ~20%.

Before infinite density is reached, other physical mechanisms will halt the collapse, then causing the cluster as a whole to expand on a timescale of $\sim 10 t_{\text{rh}}$ (refs 3,10). The core adjusts itself to supply the required energy, in much the same way that the nuclear energy sources in the Sun are regulated by energy loss from the photosphere². Thus the time-averaged energy generation rate in the core is independent of the mechanism. The more efficient the energy generation mechanism, the lower is the central density and the larger is the core radius. The mechanisms responsible for core 'bounce' and for post-collapse expansion need not be the same.

If a globular cluster can be considered as a system of point masses, energy is produced by binaries formed in three-body encounters and by subsequent interactions of these binaries with

single stars^{11–13}. This mechanism is, however, very inefficient, and requires extremely small, dense cores both at core bounce and in post-collapse¹⁴. Because of the finite radii of non-degenerate stars, however, $\sim 10^3$ binaries will form by tidal capture before the bounce¹⁵. As these binaries are nearly in contact, subsequent interactions with single stars are likely to lead to mergers (refs 16, 17 and P. W. Cleary and J. J. Monaghan, preprint). Because of their higher mass, stars produced in such mergers evolve rapidly, and then shed mass in the form of stellar winds which escape the core or even the cluster. By diminishing the binding energy of the core, this process indirectly supplies energy to the cluster. The required mass loss rate is ~1% of the total cluster mass every t_{rh} (refs 3, 10, 15). If this mass loss rate is associated with normal stellar evolution, the optical luminosity of the (small) core is comparable to that of the whole cluster, in contradiction with the observation³.

There are several ways out of this problem. Merging of main-sequence stars with white dwarfs or neutron stars may cause significant instantaneous mass ejection¹⁸. Alternatively, the cluster could contain an extremely dense core of compact objects, which generate energy by three-body formation of binaries¹⁹; but the energy production by tidal binary formation and mergers with normal stars will still be appreciable¹⁹. A black hole of mass $\leq 10^3 M_{\odot}$ would liberate binding energy by swallowing stars²⁰ and would support a large core, but it is not clear how such a black hole could form in a globular cluster.

A more conservative solution to the problem is to assume a modest population of primordial binaries. Recent radial-velocity surveys demonstrate that >1% of all giant stars in globular clusters are members of binaries, and suggest a total binary population of >10% by mass⁴. Previous work has shown that primordial binaries are ineffective in halting core collapse²¹; their importance in powering post-collapse expansion, however, has scarcely been considered.

The energy production rate per unit volume is

$$\epsilon = \left[f A_{\text{bs}} n_{\text{s}} n_{\text{b}} + \frac{1}{2} f^2 A_{\text{bb}} n_{\text{b}}^2 + 2f(1-f) A_{\text{bs}} n_{\text{b}}^2 \right] \frac{G^2 m^3}{v_{\text{s}}} \quad (1)$$

where n_{s} and n_{b} are the number densities of single and double stars; m is a typical mass of a single star; v_{s} is the one-dimensional velocity dispersion of single stars; f is the fraction

of binaries that are hard^{11,12}, that is, have an internal velocity $\geq v_c$; and A_{bs} and A_{bb} are dimensionless efficiency factors for energy production in binary-single and binary-binary encounters. For equal-mass stars, $A_{bs} = 3.8$ (ref. 22), and we estimate $A_{bb} \approx 10$ with an uncertainty of about a factor of two^{15,23,24}. The third term in equation (1) represents encounters between binaries of very different hardness, in which the softer partner is treated as an unbound pair²¹.

Mass segregation will concentrate the binaries in the core. If they dominate the central density, the total energy production in the cluster, $\dot{E}_c$, is

$$\dot{E}_c = 2.5 r_c^3 \frac{G^2 m^3}{v_c} \left[\frac{1}{2} f^2 A_{bb} + 2f(1-f) A_{bs} \right] n_b^2(0) \quad (2)$$

The energy generation is concentrated in the core and therefore is proportional to the core volume, with the core radius r_c defined²⁵ as the distance from the centre to where the projected density drops by about a factor of two:

$$r_c = \frac{3v_c}{\sqrt{16\pi G m n_b(0)}} \quad (3)$$

with v_c the central value of v_s ; we have assumed equipartition, so that the central velocity dispersion of the binaries is $v_c/\sqrt{2}$ if their mass is $2m$.

In equilibrium, $\dot{E}_c$ must supply the power needed for the expansion of the cluster, which is³

$$\dot{E}_h = \frac{|E|}{\gamma t_{rh}} \approx \frac{1}{5\gamma} \frac{GM^2}{t_{rh} r_h} \quad (4)$$

$\dot{E}_h$ is determined by two-body relaxation near the radius containing half the cluster mass, r_h . The half-mass relaxation time t_{rh} is²⁶

$$t_{rh} = \frac{0.06 M^{1/2} r_h^{3/2}}{G^{1/2} m \log_{10}(0.4 N_s)} \quad (5)$$

where M is the total mass of the cluster and $N_s \approx M/m$ is a measure of the total number of single stars in the cluster. Both self-similar and fully time-dependent Fokker-Planck calculations^{10,15,27,28} show that $\gamma \approx 10$. From equations (4) and (2) we find

$$\frac{r_c}{r_h} = \frac{0.017}{\log_{10}(0.4 N)} \left(\frac{1}{2} f^2 A_{bb} + 2f(1-f) A_{bs} \right) \left(\frac{v_c}{v_h} \right)^3 \left(\frac{\gamma}{10} \right) \approx 0.020 \quad (6)$$

The half-mass velocity dispersion, v_h , is defined by $3v_h^2/2 = GM/5r_h$. For the final estimate, we have assumed $N = 10^6$, $\gamma = 10$, $v_c = \sqrt{2}v_h$ (ref. 14), $f = 0.35$ and $A_{bb} = 10$, $A_{bs} = 3.8$. Notice that the final result does not depend on the total number of binaries in the cluster provided that they are numerous enough to predominate in the core, that is, that they constitute at least a few per cent by mass of the cluster. But the result does depend on the fraction f of binaries that are hard. In estimating $f \approx 0.35$ we have assumed that the semi-major axes of the binaries are logarithmically distributed²⁹ between $a_1 = R_\odot$ and $a_2 = 0.1$ pc, and that the hard ones are those with $a < 1$ AU—but in practice f is quite uncertain.

Equation (6) suggests that r_c may be large enough to be resolved, even from the Earth's surface. As $r_h \approx 5$ pc is a typical value for globular clusters, the corresponding angular radius of the core, if placed at the distance of the Galactic Centre, is $\sim 2''$, which is perhaps larger than is seen in many clusters^{1,30}. But the quantities in equation (6) are uncertain, and furthermore we have neglected the effects both of a mass spectrum and of gravothermal oscillations. The former will demand more elaborate modelling; the latter we shall discuss briefly.

The energy balance that we have assumed is likely to be unstable. Very large fluctuations in central density (gravothermal oscillations³¹) occur in many theoretical models of post-collapse

clusters with a variety of central energy sources^{14,31-34}. But the primordial binary source is probably even less stable than many others, for the following reason. If the core departs from equilibrium and begins to collapse, then it loses energy to the surrounding stars at a rate proportional to the ratio of the binding energy of the core to the central relaxation time⁷. This ratio scales with $n_b(0)$ and v_c in the same way as does the energy production rate (equation (4)), apart from a factor of $\log N_c$, where N_c is the number of stars in the core³⁵. Thus as the core goes out of equilibrium, its energy production cannot increase fast enough to stop the collapse, at least not before other energy-producing processes become important or N_c becomes quite small. This scaling problem may explain why primordial binaries have been found not to prevent the initial core collapse²¹. Even in the presence of oscillations, however, the time average of $\dot{E}_c$ must balance $\dot{E}_h$. Furthermore, for an energy production rate per unit volume scaling as the density squared, the time-averaged rate is not dominated by the periods of greatest central density.

Additional energy sources may be important, and some of these may indeed be most effective at times of greatest central density (as is the case with three-body production of binaries). Allowing for such sources can only increase our estimate of the time-averaged core radius. To reduce that estimate, primordial binaries must be either much less abundant or much less effective than we have supposed.

A lower present-day abundance would result not only from a lower initial abundance but also perhaps from the destruction of binaries. Energy-producing reactions will deplete the binary population, as the softer partner in a binary-binary collision tends to be disrupted. We can estimate the lifetime of the binaries from the average energy liberated per disrupted hard binary in its whole lifetime, $\nu m v_c^2$; we estimate that $\nu \approx 10$. The energy supplied to the cluster by binaries in the time, t_{depl} , required to deplete the hard binary population by a factor of $\sim e$ is $\sim 0.5 \nu M_b v_c^2 \approx 2\nu (M_b/M) |E_h|$, where M_b is the initial mass in the form of hard binaries, and $E_h \equiv -GM^2/5r_h$. The (negative) total energy of a post-collapse cluster decreases by $\sim e$ in a time $\gamma t_{rh} \approx 20$ Gyr, and thus we estimate

$$t_{depl} \approx 20 \text{ Gyr} \left(\frac{M_b}{0.05 M} \right) \left(\frac{\nu}{10} \right) \quad (7)$$

Thus we estimate that $\geq 5\%$ of the initial cluster mass should be in the form of hard binaries to maintain a post-collapse cluster at the relatively large core radius given by equation (6) for a time comparable with the age of the Universe. Depletion of binaries by disruption therefore appears to be important, but to be not rapid enough to alter our estimates by more than a factor of two. On the other hand, hydrodynamical effects (collisions) between stars may reduce the efficiency of binary interactions and increase the depletion rate (refs 16 and 17; P. W. Cleary and J. J. Monaghan, preprint; and P. J. T. Leonard, preprint).

Thus primordial binaries are likely to be important in the post-collapse evolution of globular clusters. Just how important they are depends on two uncertain quantities: their initial abundance and the rate of their destruction. Our knowledge of the first is steadily being improved by observations; as to the second, our estimates of ν and t_{depl} will be improved by numerical simulations, which are now in progress. $\square$

Received 27 February; accepted 21 March 1989.

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ACKNOWLEDGEMENTS. We thank D. Heggie and L. Spitzer for their helpful criticisms of an early draft. J. G. was supported by a Sloan Research Fellowship and a David and Lucille Packard Foundation Fellowship.

Oxygen-17 NMR in solids by dynamic-angle spinning and double rotation

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It is widely lamented that despite its unqualified success with spin-1/2 nuclei such as ^{13}C , ^{29}Si and ^{31}P , the popular NMR technique of magic-angle spinning (MAS) has experienced a somewhat restricted applicability among quadrupolar nuclei such as ^{17}O , ^{23}Na and ^{27}Al (refs 1–3). The resolution in the central ($1/2 \leftrightarrow -1/2$) transition of these non-integer quadrupolar spins under MAS is thought to be limited primarily by second-order quadrupolar broadening. Such effects of second-order spatial anisotropy cannot be eliminated by rotation about a fixed axis or by multiple-pulse techniques^{4,5}. More general mechanisms of sample reorientation (refs 6–8 and A. Samoson and A. Pines, manuscript in preparation) can, however, make high-resolution NMR of quadrupolar nuclei feasible. MAS is implemented by spinning a sample about a single axis so that second-rank spherical harmonics (which give rise to first-order broadening through anisotropy of electrical and magnetic interactions) are averaged away. But dynamic-angle-spinning (DAS) and double-rotation (DOR) NMR involve spinning around two axes, averaging away both the second- and fourth-rank spherical harmonics, which are responsible for second-order broadening. Here we present the application of these new techniques to ^{17}O in two minerals, cristobalite (SiO_2) and diopside ($\text{CaMgSi}_2\text{O}_6$). This work goes beyond previous results on ^{23}Na (ref. 8) by showing the first experimental results using DAS and by demonstrating the application of DOR to the resolution of distinct oxygen sites in an important class of oxide materials.

Both cristobalite and diopside have been studied by ^{17}O NMR using conventional sample-spinning techniques (ref. 9 and E.

Oldfield and R. J. Kirkpatrick, manuscript in preparation). Cristobalite is known to have a single crystallographically distinct oxygen site, with the oxygens arranged tetrahedrally about each silicon atom¹⁰. Crystalline diopside, shown in Fig. 1, contains three distinct oxygen sites¹¹ which give rise to overlapping MAS powder patterns even for high NMR magnetic fields (~ 10 tesla). E. Oldfield (personal communication) has pointed out that, under conditions of MAS, magnetic fields in excess of 25 tesla might be required to clearly resolve resonances from the three oxygen sites, as illustrated in Fig. 2.

In dynamic-angle spinning, the spinning axis is flipped from an angle θ_1 to an angle θ_2 after a given length of time. Figure 3 demonstrates the application of DAS to a sample of cristobalite enriched to 37% in ^{17}O . The experiment was performed at 54.25 MHz, allowing evolution for a time $t_1/2$ while the sample spun at 3,040 Hz around an axis inclined at $\theta_1 = 37.4^\circ$ and a further time $t_1/2 + t_2$ after the spinning axis had been flipped to

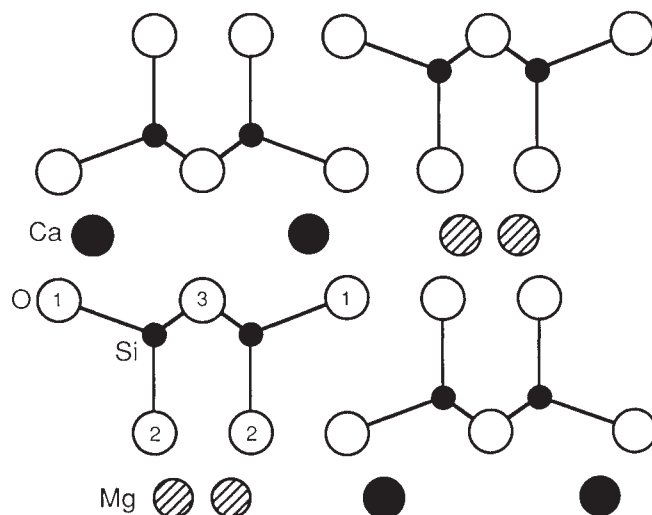


FIG. 1 Structure of diopside, $\text{CaMgSi}_2\text{O}_6$, viewed as a projection along the tetrahedral chains. Oxygen is represented by open circles with the three distinct sites labelled.

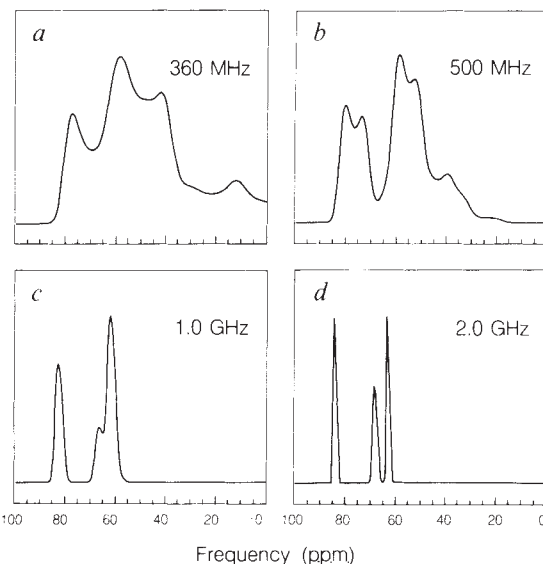


FIG. 2 Simulations of ^{17}O magic-angle-spinning spectra for diopside at magnetic fields (8.4–47 tesla) corresponding to proton NMR frequencies of 360 to 2,000 MHz (E. Oldfield, personal communication).

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an angle of $\theta_2 = 79.2^\circ$ (ref. 6). During the axis flip (~ 35 ms), magnetization is stored along the magnetic field^{12,13}. Phase-cycling followed by Fourier transformation yields a two-dimensional plot with the ω_1 frequency axis corresponding to a DAS spectrum devoid of second-order quadrupolar broadening. A further DAS experiment with the sample spinning at 2,460 Hz identifies a single centre-band of linewidth ~ 200 Hz, two orders of magnitude narrower than the central transition of ^{17}O in a static sample of cristobalite.

In the double-rotation experiment the sample is spun around two axes inclined at angles corresponding to zeros of the second and fourth Legendre polynomials, $\theta^{(2)} = 54.7^\circ$ and $\theta^{(4)} = 30.6^\circ$ respectively. Rapid sample rotation about these 'magic angles' results in removal of first-order ($\theta^{(2)}$) and second-order ($\theta^{(4)}$) broadening. For a sample of diopside enriched to 20% in ^{17}O , the MAS spectrum in Fig. 4a collapses under DOR, using a new Berkeley-Tallinn (BETA) double rotor (Y. Wu, A. Samoson and A. Pines, manuscript in preparation), at an excitation frequency of 54.25 MHz to three resolved peaks of linewidth ~ 100 Hz (Fig. 4b, c). The ^{17}O peaks in Fig. 4b, c are easily assigned to the three sites of diopside shown in Fig. 1.

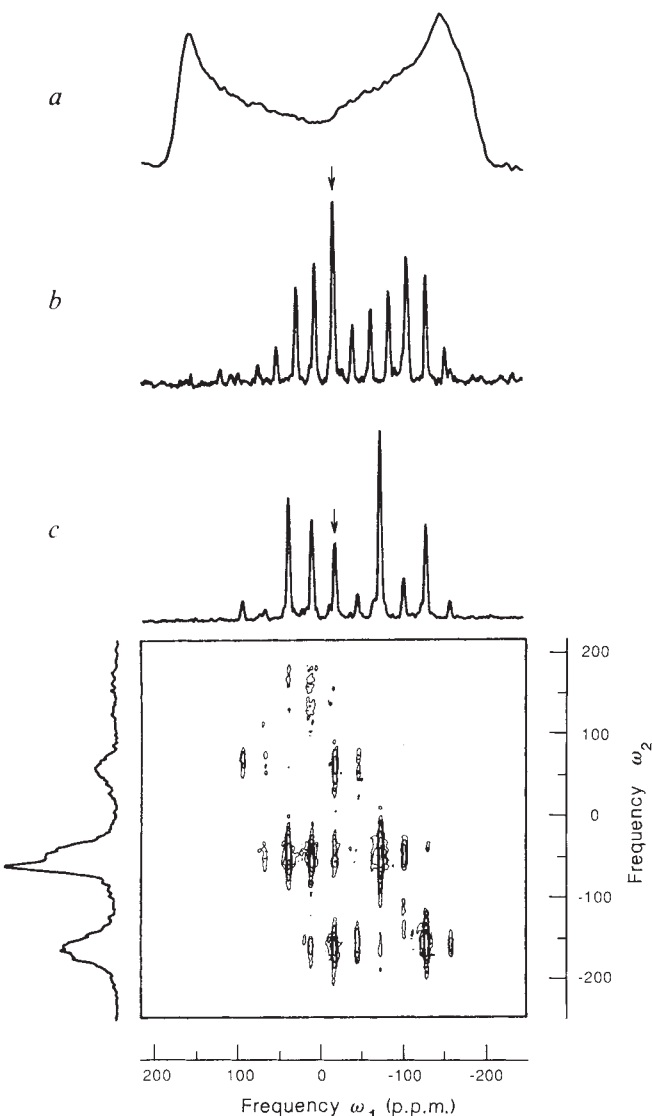


FIG. 3 NMR spectra of ^{17}O in cristobalite, SiO_2 , with a static sample (a), and under conditions of dynamic-angle spinning (DAS) at 2,460 Hz (b) and 3,040 Hz (c), showing a single centre-band peak of linewidth ~ 200 Hz. The vertical frequency axis (ω_2) in the two-dimensional spectrum corresponds to the sample spinning at an angle of $\theta_2 = 79.2^\circ$. Isotropic-shift scale referenced to H_2^{17}O .

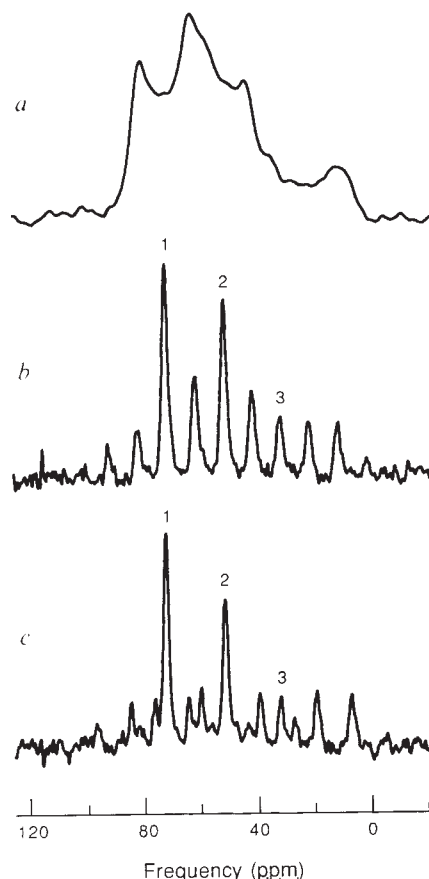


FIG. 4 NMR spectra of ^{17}O in a sample of diopside, $\text{CaMgSi}_2\text{O}_6$, under conditions of magic-angle spinning (MAS) (a), and in response to double rotation (DOR) with the outer rotor spinning at 540 Hz (b) and 680 Hz (c). The isotropic centre-band peaks correspond to the numbered oxygen sites in Fig. 1. Isotropic-shift scale referenced to H_2^{17}O .

Thus DAS and DOR do indeed seem to provide much enhanced resolution of isotropic chemical and second-order shifts for quadrupolar nuclei such as ^{17}O . We anticipate that, together with zero-field and SQUID magnetic resonance^{14,15}, these NMR techniques will contribute to the understanding of microstructural oxygen environments in solids and should constitute invaluable tools in the study of oxide materials in, for example, mineralogy, catalysis and high-temperature superconductivity. □

Received 8 February; accepted 21 March 1989.

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ACKNOWLEDGMENTS. We thank M. Berger, A. Samoson and B. Q. Sun for advice, and H. Graetsch and K. Gaskins for help in machining the ^{17}O probes. This work was supported by the Office of Energy Research, Office of Basic Energy Sciences, Materials and Chemical Sciences Division of the US Department of Energy.

Late Quaternary palaeoclimatic oscillations in East Africa recorded by heavy minerals in the Nile delta

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BECAUSE the Nile carries a load derived from markedly different geological terrains and climatic zones, it is expected that mineralogical study of deposits in its delta could help define changes in palaeoclimate affecting East Africa. Here we report measurements on heavy minerals in the Nile delta which can be used as distinct markers of climatic shifts over Africa during the late Pleistocene to Recent. Variations of heavy minerals in radiocarbon-dated cores collected in this delta¹, the Nile's major depocentre², correlate closely with oscillations in African palaeoclimate determined by other independent methods, such as studies of lake levels. These variations are largely a function of the characteristics of the Nile. It flows across nearly 35° of latitude, from south of the Equator to the Mediterranean (Fig. 1a), and conditions in its drainage basin, which includes central Africa, the Ethiopian plateau and eastern Sahara, range from a tropical, humid to a warm, arid climate. East African climatic belts migrated considerably during the Quaternary³⁻⁵, modifying Nile discharge and sediment load. Climatic oscillations have been recorded by changes of sediment yield and grain size along its course^{6,7} and by well defined depositional cycles⁸, including sapropels⁹, in the eastern Mediterranean.

The Main Nile, north of 18° N latitude, comprises flow from three major tributaries: the White Nile, Blue Nile and Atbara river (Fig. 1b). At present, their hydrology and sediment load are markedly different¹⁰ (Fig. 2). The White Nile contributes almost a third of the total discharge, but only a minor proportion of sediment; pyroxene is almost absent, whereas amphibole, eroded from metamorphic terrains, accounts for a high proportion of the transparent heavy minerals suite¹¹. The Blue Nile contributes more than half of the Main Nile discharge and almost three-quarters of the sediment load; the proportion of pyroxene relative to amphibole is high¹¹. The Atbara contributes only a small part of the total discharge but one quarter of the Main Nile sediment load; pyroxene is much more abundant than amphibole¹¹. Differences in discharge of these three tributaries are a function of different surface areas of their respective drainage basins and differences in rainfall (Fig. 1a, b). Differences in sediment load record seasonal variations in rainfall^{10,12} and vegetal cover¹³ in the various basins. Differences in composition of sediments in the three tributaries are due to markedly different geological formations eroded in the Ethiopian highlands and central African plateau¹⁴.

In contrast to the negligible amounts of pyroxene carried by the White Nile is the high proportion of pyroxene in the Blue Nile and even higher proportions in the Atbara. These high proportions result from erosion of Ethiopian volcanic terrains. Pyroxene and amphibole, with a roughly comparable specific gravity and weathering stability index¹⁵, generally constitute more than 50% of the total heavy mineral suite in Main Nile samples. It should thus be possible to evaluate changes in the sediment load contributed by each of the three main tributaries by studying the relative proportions of these heavy minerals carried by the Main Nile. The relative proportion of amphibole to pyroxene has been calculated using a standardized index (ratio) applied to compare deposits in the various Nile river tributaries. This index was defined by Hassan¹⁶: $I_{Amph} =$

(frequency of amphibole/frequency of amphibole + pyroxene) × 100. Sand fractions were selected for study from 73 core samples of late Pleistocene and Holocene age in the Lake Manzala region of the eastern Nile delta (Fig. 1c), including cores S6, S7, S8 and S22^{1,17}. Cores S7 and S22 are somewhat coarser and provide larger numbers of samples (29 from S7, and 28 from S22) containing heavy minerals of sand size grains coarser than 63 µm. At least 300 heavy mineral grains (63–250 µm in size) were identified in each sample using standard petrographic microscope techniques. We have prepared a tabulated list of heavy mineral percentages and I_{Amph} values for all studied core samples (available from the authors).

Core S7 is stratigraphically more complete and comprises four major stratigraphic units, from the base upwards (Fig. 3): IV—fluvial sands, 24.5–19.3 m from the core top; III—oxidized silty muds of interchannel, sebkha and/or swamp origin, 19.3–14.7 m; II—fluvial and coastal sands, 14.7–10 m; and I—marine delta-front muds, coastal sands (5–4 m) and subaerial deltaic sequences at the core top. Unit I represents the recent progradational delta. Radiocarbon dates indicate that units IV, III and II are upper Pleistocene (IV and III from about 40,000 to 20,000 yr BP; II from about 20,000 to 12,000–10,000 yr BP). Unit I, mid- to upper-Holocene, ranges from about 8,000 yr BP to the Present. A depositional hiatus occurs between 12,000–10,000 and 8,000 yr BP¹⁷.

The relative proportions of amphibole and pyroxene, as measured by the I_{Amph} index, vary considerably from the base to the top of core S7 (Fig. 3). In units IV and III, I_{Amph} values average 63.4 ± 5 (numbers after $\pm$ refer to 1 standard deviation). In unit II, the index is 35.7 ± 7 . In unit I, index values increase initially (76.8 ± 20 between 8.2 and 5.8 m; maximum value of 96), and then decrease markedly further up (38.0 ± 11 between 5.5 m and the core top). Note that there is no correlation between I_{Amph} values and specific lithofacies or grain size, indicating that the proportion of the two minerals in this core is controlled by factors other than local hydrodynamic processes.

Core S22, longer than S7, comprises a thicker Holocene section (unit I) but does not include unit IV (Fig. 3). Unit III forms the base (from 38.8 to 37.7 m) and unit II occurs from

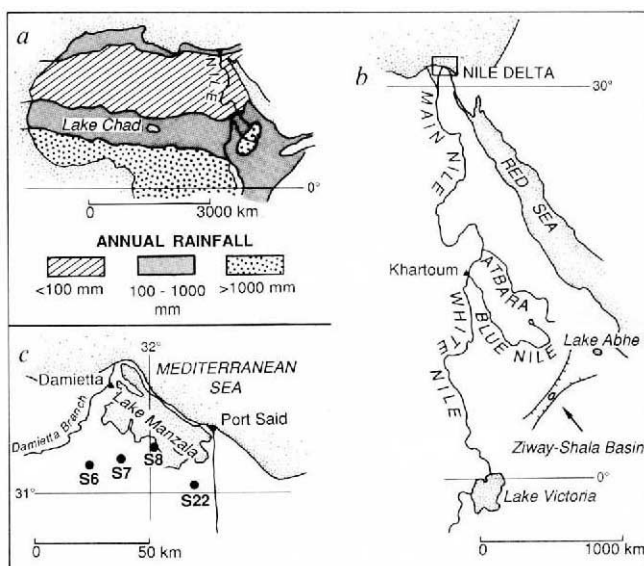


FIG. 1 a, Map showing position of the Nile river and Lake Chad, and annual rainfall on the African continent¹³. b, Map showing the Nile delta, Nile river and its tributaries, the Atbara and Blue Nile draining the Ethiopian plateau and White Nile draining the Central African plateau. Box indicates area in c. c, Map of the north-eastern Nile delta, showing the position of studied cores.

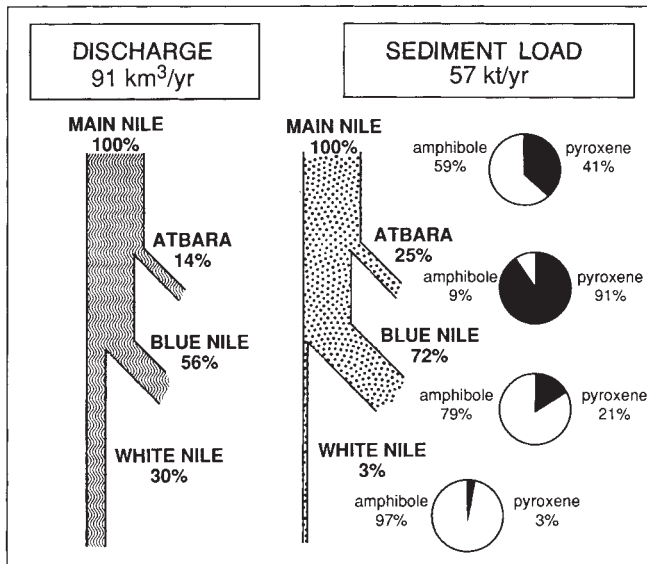


FIG. 2 Diagrams showing (left) the total Nile river discharge and relative proportions supplied from its three main tributaries, and (right) total sediment load and relative load proportions in the tributaries (compare ref. 10). Pie diagrams indicate the relative proportions of amphibole and pyroxene in the present bed-load of the Main Nile and its tributaries¹¹.

37.7 to 22 m; unit I constitutes more than half of the core length. A sand layer between 5 m and 3.2 m probably correlates with the sand section at 5–4 m in core S7. In unit II, *I*Amph index values average 29.6 ± 6 . In unit I, heavy minerals are absent in fine-grained muds between 22 m and 15 m; above this, *I*Amph values are initially low (29.7 ± 13 between 15 and 8 m) but then increase (67.2 ± 13 ; maximum value of 84.5) between 8 and 4.5 m. Deposits at the top of the core reveal an *I*Amph index of 44.2 ± 9 . The overall pattern of the *I*Amph values parallels that in core S7; some differences may be due to local reworking by coastal currents on the delta margin to cause removal of less dense minerals and concentrate opaque heavy minerals¹⁸, which occurred, for example, to an extent of >75% between 14.5 and 6 m.

There are fewer sandy samples with heavy minerals in cores S6 and S8¹⁹, but *I*Amph index patterns in these two cores are consistent with those of time-equivalent sections in S7 and S22.

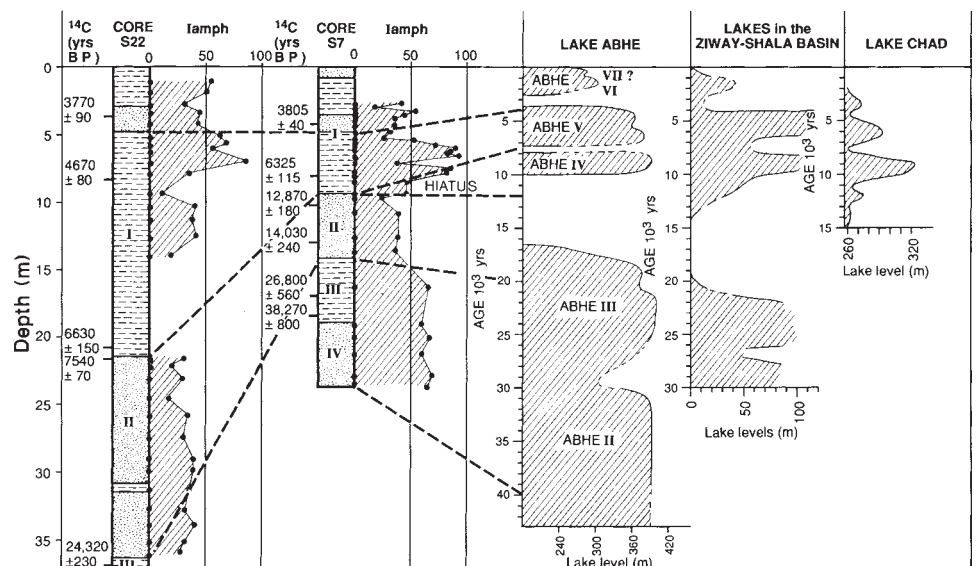
Also calculated are *I*Amph values in sandy samples from two suites of cores further to the west in the Nile delta, in the Damietta and Rosetta promontories²⁰. Values in these cores, available only for the upper parts of units II and I, are comparable to those of cores S7 and S22.

Patterns of *I*Amph values are not random but vary during specific time periods (Fig. 3). We attribute these distinct time-related variations to changes in the relative sediment load contributions from major Nile tributaries. Studies of time-related changes of sediment yield and grain size during the late Quaternary⁷ show that the load of the Blue Nile and Atbara, both primary sources of pyroxene in the Main Nile, varied considerably during this period. The large load presently carried by these two rivers results from the interplay of seasonal, and often intense, rainfall (primarily during summer) affecting an arid region with moderate to low vegetation cover over most of the Ethiopian drainage basin area^{10,12,13}. It is probable that more humid phases affecting this region would have given rise to a longer rainy season and denser vegetation cover. An increased vegetal cover reduces erosion and removal of sediments. Thus, wetter conditions would have resulted in decreased sediment loads carried by both Ethiopian tributaries, even if their discharge were to have increased⁷. This decreased sediment load would result in a decreased supply of pyroxene to the Main Nile and, in consequence, an increased *I*Amph value. This increase would have been particularly important at times of extended vegetal cover over the Atbara drainage basin, the main source of pyroxenes.

White Nile load fluctuations are also affected by changing climatic/geographical factors, and so would also have induced changes in proportions of heavy minerals, for example during times of cut-off from Uganda headwaters before ~12,500 yr BP⁶. Even if the discharge of the White Nile were to have increased considerably, its load would have remained generally low because of a more constant vegetation cover on its drainage basin during most of the late Quaternary^{21,22}. The White Nile sediment contribution became relatively much more important when loads of the Blue Nile and Atbara decreased during humid phases. Simplified calculations using the Nile hydrological data in Fig. 2 suggest, for example, that *I*Amph values of ~80 imply equivalent sediment loads from the White Nile and Ethiopian tributaries.

Thus, decreased proportions of pyroxene (that is, an increase in *I*Amph values) in sediments of the Main Nile and in the delta would indicate an increased vegetation cover in the drainage basins of the Blue Nile and the Atbara, and consequently,

FIG. 3 *I*Amph index patterns plotted along radiocarbon-dated lithostratigraphic sections of cores S22 and S7. I to IV corresponds to stratigraphic units discussed in text. Time correlation indicated by dashed lines. *I*Amph patterns are correlated with lake level variations, including those of Lake Abhe²³, lakes in the Ziway-Shala²⁴ Basin and Lake Chad²⁵.



more humid conditions. At such times it is also probable that tributaries (now dry), draining extensive areas and coming in from the desert along the western flank of the Main Nile, yielded more amphibole than pyroxene (D. A. Livingstone, personal communication). On the other hand, increased proportions of pyroxene transported to the Main Nile (that is, a decrease in *I*Amph values) would probably indicate a reduction of vegetation cover, accentuated erosion of the Ethiopian plateau and thus more arid conditions. Moreover, some fluctuations recorded in cores probably reflect temperature and rainfall oscillations. Lower temperatures may cause an increase in loads contributed by the Atbara and Blue Nile as a result of solifluction, lowered tree-lines and increased hillside-slope instability (M. A. J. Williams, personal communication).

These interpretations can be evaluated in the light of late Quaternary palaeoclimatological studies that use other techniques, such as the measurement of African lake levels (Fig. 3). The temporal pattern of the *I*Amph index in core S7 correlates, for example, with changes of level of Lake Abhe east of the Ethiopian plateau²³, and lakes in the Ziway-Shala Basin in the Ethiopian Rift²⁴ (Figs 1b and 3). High *I*Amph values in units IV and III (~40,000–20,000 yr BP) correspond to periods of high lake levels recorded before about 20,000–17,000 yr BP. Low *I*Amph values in unit II (~20,000 to 12,000–10,000 yr BP) correlate with low lake levels from about 20,000–17,000 to 14,000–10,000 yr BP. *I*Amph values which increase and then decrease upward in unit I, between 10 and 5.5 m from the core top, span a period of high lake levels between 7,000 and 4,000 yr BP. The increased *I*Amph values at ~3.4 m may be related to the climatic phases that induced high lake levels found for ~1,500 yr BP.

In summary, heavy minerals suites in dated Quaternary Nile delta deposits serve not only as provenance markers^{11,19} but also as valuable indicators of East African palaeoclimatic oscillations. Pronounced time-related changes in proportions of pyroxene are directly related to climatic oscillations over the Ethiopian plateau, and to very large changes in sediment loads of major Nile tributaries. These changes were probably associated with larger-scale north-south displacements of climatic belts across extensive sectors of the African continent. This is supported by correlations of Nile delta compositional data with variations of lake levels in distant regions, such as Lake Chad²⁵ (Fig. 3) located at about the same latitude and about 3,000 km west of the Ethiopian plateau (Fig. 1a). Study of heavy minerals in dated sedimentary sequences holds promise as an independent criterion to be used in conjunction with other methods to interpret regional palaeoclimatic oscillations. □

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ACKNOWLEDGEMENTS. We thank Mr H. Sheng for assistance with the heavy mineral analyses, and Drs H. R. Davis, H. Faure, D. A. Livingstone, M. Servant and M. A. J. Williams for their reviews. This study, part of the Mediterranean Basin (MEDIBA) Program (D.J.S.) and "Evolution des climats et sédimentation" program (A.F.), was funded by grants from the Smithsonian Scholarly Studies Program, AMOCO Production Company, the National Geographic Society and the Muséum national d'Histoire naturelle.

Molecular evidence for pre-Cretaceous angiosperm origins

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FLOWERING plants or angiosperms have dominated the Earth's flora since at least the late Cretaceous¹ and were already highly diversified by Barremian times, about 120 million years (Myr) ago. However, because of the paucity of fossilized angiosperm reproductive structures from lower Cretaceous sediments^{2,3} and the absence of generally recognized angiosperm fossils from pre-Cretaceous strata^{4,5}, their origins and early evolution remain obscure. Similarly, attempts to understand pre-Cretaceous angiosperm evolution^{4–11} have been impaired by difficulties in defining and interpreting angiospermous characters in fossil specimens^{8,12}. We report here molecular evidence suggesting that angiosperm ancestors underwent diversification more than 300 Myr ago.

To obtain a molecular view of angiosperm origins we have determined nucleotide sequences for full-size complementary DNAs of a slowly evolving glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), from six flowering plants. The six nucleotide sequences from animals^{13,14}, one from yeast¹⁵, and nine from plants (this study and refs 16–18) yielded, upon alignment, 332 codons for comparison in each of the 16 coding regions. Divergence was measured with the weighted pathway method¹⁹. Pairwise comparisons between organisms for which divergence times are roughly known reveal that the nonsynonymous substitution rate (K_a) of GAPDH has remained constant along separate eukaryotic lineages (Table 1). Were the nonsynonymous rate for GAPDH within angiosperms or their antecedents markedly accelerated relative to that in animals, we would expect values of K_a for comparisons involving plants to exceed those for animals versus yeast. Yet the yeast outgroup reveals that the nonsynonymous rate for GAPDH in plants may be slightly (7%) lower than that in animals. Eukaryotic GAPDH sequences are at compositional equilibrium²⁰ for first and second codon positions (data not shown); values of K_a in comparisons between angiosperm GAPDH sequences should thus provide reasonable estimates for the timescale of angiosperm evolution.

Numbers of nonsynonymous and synonymous substitutions per site (K_a and K_s , respectively) for comparisons between angiosperm GAPDH sequences are shown in Table 2. Included in the table are the corresponding values for comparisons of full-size complementary DNA sequences for chalcone synthase (CHS), the key enzyme of anthocyanin biosynthesis, from seven²¹ of the nine angiosperms considered here. The parallel analysis of enzymes from primary (GAPDH) and secondary (CHS) metabolic pathways contributes significantly to the elimination of potential errors inherent in phylogenetic inferences based upon a single gene. For these two nuclear genes and the seven species from which both sequences have been determined, average divergence at synonymous and nonsynonymous sites between monocots and dicots consistently exceeds that within

Received 28 November 1988; accepted 9 March 1989.

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dicots, congruent with observations for plant organellar DNA sequences^{22,23}. These findings suggest that no comparisons between monocots and dicots for GAPDH or CHS involve genes which were duplicated before the monocot-dicot separation.

Divergence at nonsynonymous sites between angiosperm GAPDH and CHS cDNAs and the implications for the course of angiosperm evolution are summarized in Fig. 1. This general picture of angiosperm evolution is quite distinct from that for which the fossil record can provide clear evidence. The time of divergence indicated for the monocot-dicot separation pre-dates the appearance of generally recognized angiosperm fossil remains by roughly 200 Myr. The deepest branch of the topology does not separate *Magnolia* from the remaining angiosperms²⁴, but rather monocotyledons from dicotyledons.

Numbers of synonymous substitutions per synonymous site, K_s (ref. 19), were determined for GAPDH and CHS cDNAs (Table 2) and set in relation to the timescale in Fig. 1 for estimation of synonymous rates in these two plant nuclear genes. Based on this timescale, the average synonymous substitution rate for angiosperm GAPDH from 35 pairwise comparisons is 2.3×10^{-9} per site per year, which is very close to that for GAPDH in the mammalian comparison (2.4×10^{-9} per site per year). The synonymous rate for GAPDH of angiosperms and mammals is then lower than the average for mammalian genes, yet this is not surprising in light of the generally positive correlation found between synonymous and nonsynonymous rates¹⁹. The average synonymous rate obtained for chalcone synthase from 12 total pairwise comparisons in which the method could effectively correct for multiple substitutions is 4.9×10^{-9} per site per year. This value is in good agreement with the average synonymous rate¹⁹ for mammalian genes (4.7×10^{-9}). Thus, if the timescale depicted in Fig. 1 is approximately correct, the present data would suggest that nuclear synonymous rates in angiosperms and mammals are very similar.

There are several alternative hypotheses to account for the sudden appearance of highly diversified angiosperm taxa in lower Cretaceous sediments^{4-12,25-27}. Evidence supporting the

TABLE 1 Numbers of nonsynonymous substitutions in glycolytic GAPDH nucleotide sequences from eukaryotes

	<i>n</i>	K_a	s.e.	Rate
Plants-animals	54	0.266	(0.021)	0.133×10^{-9}
Plants-yeast	9	0.269	(0.022)	0.135×10^{-9}
Animals-yeast	6	0.287	(0.023)	0.144×10^{-9}
<i>Drosophila</i> -vertebrates	6	0.181	(0.017)	0.150×10^{-9}
Mammals-chicken	2	0.047	(0.008)	0.088×10^{-9}
Human-rat	1	0.030	(0.006)	0.179×10^{-9}

Numbers of nonsynonymous substitutions (K_a) in glycolytic¹⁶ GAPDH nucleotide sequences from eukaryotes. Values of K_a represent the average of mean values¹⁹ for the number of pairwise comparisons given in column *n*. The average of standard errors (s.e.) over comparisons are indicated in brackets. Rates are expressed as numbers of substitutions per site per year and were calculated with the following divergence times^{1,3,28,29}: plants-animals-yeast: 1,000 Myr; *Drosophila* (two genes)-vertebrates: 600 (Myr); mammals-chicken: 270 Myr; human-rat: 85 Myr. The comparison between mammals and chicken gives the lowest estimate of nonsynonymous substitution rate for GAPDH, consistent with observations for cytochrome *c* and globins^{28,29} in comparisons of these vertebrates. The highest rate (human-rat) is subject to the stochastic error of a single comparison involving a rather small number of total substitutions. Average values of K_a in interkingdom comparisons involving plants are lower than that for animals versus yeast, suggesting a slightly lower overall rate for the line leading to angiosperms. Using yeast as an outgroup, the relative rates test³⁰ reveals no differences in plant versus animal rates that are significant at $P=0.05$ (data not shown). The average nonsynonymous substitution rate for GAPDH from all 78 pairwise comparisons is 0.135×10^{-9} . The average nonsynonymous rate for those 15 comparisons excluding plants is 0.141×10^{-9} . The latter average nonsynonymous rate was conservatively chosen to apply to values of K_a in comparisons between angiosperms (Fig. 1) because it provides minimum estimates of angiosperm age. Although cells representing eukaryotic algae appeared 1.4–1.5 $\times 10^9$ years ago^{31,32}, the first photosynthetic eukaryotes were probably not the ancestors of higher plants³². Use of divergence times exceeding the estimate of 10^9 years for plant-animal-yeast separation would result in lower average values of nonsynonymous rate for GAPDH and thus imply ages for angiosperms greater than those indicated in Fig. 1.

TABLE 2 Numbers of nonsynonymous and synonymous substitutions for GAPDH and CHS of angiosperms

	<i>n</i>	GAPDH		<i>n</i>	CHS	
		$K_a \times 100$ (s.e.)	$K_s \times 100$ (s.e.)		$K_a \times 100$ (s.e.)	$K_s \times 100$ (s.e.)
Dicots versus monocots						
<i>Ranunculus</i> -monocots	2	9.02 (1.1)	156 (47)	4	10.6 (1.1)	NC
<i>Sinapis</i> -monocots	2	7.24 (1.0)	173 (20)			
<i>Magnolia</i> -monocots	2	7.16 (1.0)	142 (20)	2	10.5 (1.1)	NC
<i>Petroselinum</i> -monocots	2	10.5 (1.2)	177* (48)	2	11.1 (1.2)	NC
Scrophulariales -monocots	6	9.62 (1.2)	156 (25)	4	11.7 (1.2)	NC
Monocots versus monocots						
<i>Hordeum</i> - <i>Zea</i>	1	2.90 (0.6)	56 (7)	1	6.5 (0.8)	86 (17)
Dicots versus dicots						
<i>Ranunculus</i> -remaining dicots	6	7.14 (1.0)	107 (13)	10	10.1 (1.1)	281* (11)
<i>Sinapis</i> -remaining dicots	6	6.37 (0.9)	136 (23)			
<i>Magnolia</i> -remaining dicots	6	6.27 (1.0)	141 (22)	6	9.7 (1.1)	240* (55)
<i>Petroselinum</i> -remaining dicots	6	8.53 (1.0)	128 (17)	6	10.1 (1.1)	261* (26)
Scroph'ales -remaining dicots	12	6.86 (1.0)	123 (16)	12	10.6 (1.1)	289* (108)
<i>Antirrhinum</i> -Solanaceae	2	5.74 (0.9)	101 (12)	2	6.3 (0.9)	NC
<i>Nicotiana</i> - <i>Petunia</i>	1	5.60 (0.9)	114 (14)			

The table shows the numbers of nonsynonymous (K_a) and synonymous (K_s) substitutions for GAPDH and CHS of angiosperms, as determined by the weighted pathway method¹⁹ with a program kindly provided by W.-H. Li. Values of K (K_a or K_s) represent the average of mean values from the number of pairwise comparisons given in column *n*. The average standard error (s.e.) over comparisons²² is indicated in brackets. Both CHS cDNAs of *Ranunculus*²¹ and a full-size cDNA from the second active CHS gene of *Petunia* (J. Reif; unpublished data) were included in the comparisons. Cases in which the method was unable to correct for multiple substitutions at identical sites are indicated by NC. An asterisk denotes that the method was unable to correct in all possible pairwise comparisons. Scrophulariales is the common order of *Antirrhinum*, *Petunia* and *Nicotiana*, the latter two being common members of the Solanaceae. Comparisons for both genes are based upon alignments which exclude amino- and carboxy-terminal regions of length heterogeneity. An average angiosperm GAPDH pair contains 776 nonsynonymous and 220 synonymous sites. The *Nicotiana* sequence is only nearly full size¹⁸ and thus contains about 3 per cent fewer sites. An average CHS pair contains 903 nonsynonymous and 264 synonymous sites. GAPDH cDNA clones were identified by heterologous plaque hybridization to the 1.1-kb *Pst*I fragment of ps198 (ref. 17) subcloned into plasmid vectors, and sequenced by the chemical cleavage method³³. The *Petroselinum* cDNA library was provided by W. Schulz, the remaining clones were isolated from libraries previously described²¹. The published¹⁶⁻¹⁸ and six new angiosperm sequences (submitted to GenBank), their alignment to other eukaryotic GAPDH sequences, and the K_a matrix of pairwise eukaryotic comparisons are available from the authors upon request.

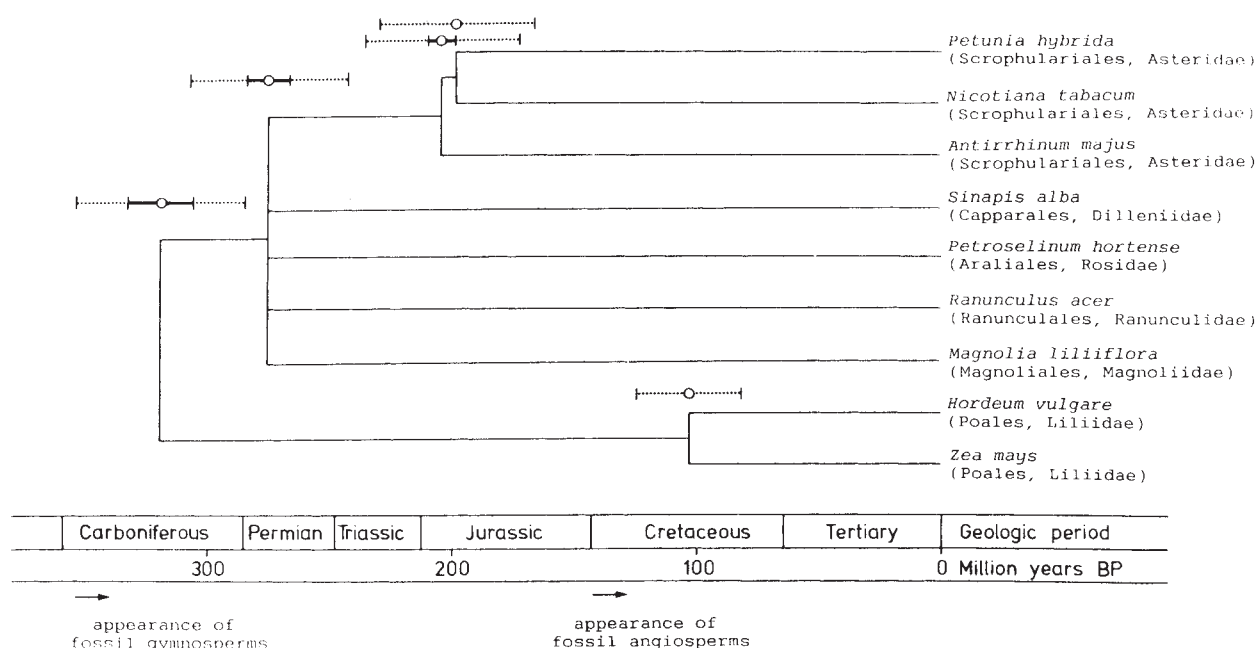


FIG. 1 A molecular view of angiosperm evolution based upon nonsynonymous substitutions in GAPDH and CHS. Order and subclass assignment²⁶ for each species is indicated in parentheses. The time of divergence between monocots and dicots was obtained by dividing the average value of K_a for GAPDH from all 14 pairwise monocot-dicot comparisons (0.0900 ± 0.0036) by twice the average nonsynonymous rate for GAPDH from Table 1 (0.141×10^{-9} per site per year). The time of subclass radiation for dicots was estimated by determining the ratios of K_a (dicots-dicots): K_a (dicots-monocots) for GAPDH and CHS for all five dicotyledonous subclasses represented (Table 1) and multiplying the average of these nine ratios (0.868 ± 0.027) with the derived divergence time for monocots-dicots. Divergence times within the Scrophulariales and for barley-maize were estimated solely on the basis of K_a and average rate for GAPDH. The geological timescale

used is that of Thomas and Spicer¹². Solid bars indicate the standard error for averages of mean K_a or, in the case of dicot subclass radiation, the standard error of monocot-dicot ratios. Dotted bars indicate the average standard error of K_a (ref. 19) across comparisons at a given node. Estimated divergence times shown are: *Zea-Hordeum* 103 ± 22 Myr; *Petunia-Nicotiana* 199 ± 32 Myr; *Antirrhinum-Solanaceae* 204 ± 32 Myr; dicotyledonous subclass radiation 276 ± 33 Myr; monocot-dicot separation 319 ± 35 Myr. Subclass assignments for these angiosperms are not identical in different classification schemes^{10,26,34}. Molecular sequence (DNAML, DNAPENNY and DNABOOT of PHYLIP³⁵, version 3.0 provided by J. Felsenstein) and matrix (FITC of PHYLIP, UPG and modified³⁶ UPG) tree-building algorithms did not yield consistent topologies for dicot subclasses, as reflected in the error bars in the figure.

view of Cretaceous angiosperm origins is of a negative nature in that lack of generally recognized angiosperm fossils from older strata is interpreted as nonexistence of the organisms in pre-Cretaceous time. The present molecular data may be interpreted as support for the theories of those who have argued in favour of a long pre-Cretaceous history for angiosperms. Palaeogeographic^{4,25} and ecological¹⁰ considerations have suggested that the lack of pre-Cretaceous angiosperm fossil remains could be explained through an adaptation of primitive angiosperms to restricted (upland) habitats, thus inhibiting fossilization up until their Cretaceous invasion of such lowland habitats as are conducive to sedimentation. The Permo-Triassic time of primary angiosperm radiation suggested by Axelrod²⁵ is easily reconciled with our results. Yet, using quite different arguments, proponents of polyphyletic angiosperm origins have also suggested that divergence between angiosperm lineages occurred in pre-Cretaceous times^{7,27}. The temporal implications and early monocot-dicot separation shown in Fig. 1 do not exclude the hypothesis that some characters viewed as angiospermous⁶ arose along independent ancestral routes. Recently, most parsimonious cladistic trees based upon morphological character states⁹ have implicated Triassic angiosperm origins, an estimate of angiosperm age which would appear too conservative in light of the divergence between angiosperm GAPDH sequences. We conclude that the synthesis of molecular and palaeobotanical findings should result in the generation of experimentally testable hypotheses concerning angiosperm phylogeny. □

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Received 23 November 1988; accepted 29 March 1989.

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ACKNOWLEDGEMENTS. We thank P. Starlinger, William Chaloner, D. Lydiate and J. Dangi for comments on the manuscript.

Stimulated activity mediates phase shifts in the hamster circadian clock induced by dark pulses or benzodiazepines

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A NUMBER of environmental and pharmacological stimuli capable of inducing phase shifts and/or period changes in the circadian clock of mammals have now been identified¹⁻³. Agents that can alter circadian clocks provide a means for investigating the cellular and neural mechanisms responsible for their generation, regulation and entrainment. Two stimuli that have been used to probe the basis of circadian rhythmicity are pulses of darkness on a background of constant light⁴⁻⁸ and injections of short-acting benzodiazepines, such as triazolam⁹⁻¹¹. Surprisingly, these two very different stimuli have remarkably similar phase-shifting effects on the circadian clock of hamsters. The observation that a short-term increase in locomotor activity occurs when the circadian activity rhythm of hamsters is shifted by dark pulses or triazolam injections^{5,6,9}, coupled with the finding that activity bouts themselves are capable of shifting this rhythm^{12,13}, raises the possibility that dark pulses or triazolam alter the circadian clock by inducing acute hyperactivity. Here we demonstrate that the phase-advancing and phase-delaying effects of dark pulses or triazolam on the circadian activity rhythm can be totally suppressed by immobilization of the animals during treatment. These results indicate that behavioural events mediate the phase-shifting effects of both dark pulses and triazolam on the circadian activity rhythm and question present hypotheses regarding the pathways by which light-dark information and pharmacological agents influence circadian pacemakers.

Nine-week-old outbred male golden hamsters (*Mesocricetus auratus*), raised in our colony from animals purchased from Charles Rivers Lakeview (Newfield, New Jersey), were initially group-housed and maintained on a 14:10 light-dark (LD) cycle (14 h of light per 24 h) until 9 weeks of age. Animals were then moved to individual cages equipped with a running wheel to allow for the continuous recording of locomotor activity⁶. After 7-16 days with the wheel, the animals were either transferred to constant light (LL, fluorescent lighting, intensity ~600 lux at cage floor) or were bilaterally enucleated under pentobarbital anaesthesia (100 mg kg⁻¹). Immediately after injection of vehicle or triazolam, or at the same time of the exposure to the dark pulse, hamsters were either left undisturbed or immobilized. All perturbations were timed relative to the onset of activity which is usually designated as circadian time (CT) 12 in this nocturnal species.

As expected, exposure to darkness or injections of triazolam in unrestrained animals at CT 6 resulted in clear phase advances in the rhythm of activity while the same treatments at CT 18 or 21, respectively, induced phase delays (Figs 1 and 2). Immobilization totally blocked both the phase advances and phase delays normally induced by exposure to a 6 h dark pulse. Similarly, no clear phase advance (>15 min) was observed in any of the restrained hamsters injected with triazolam at CT 6, and only 2 out of 14 immobilized hamsters injected with triazolam at CT 21 exhibited a phase delay greater than 15 min. At the circadian times tested, restraint alone had no phase-shifting effect on the

activity rhythm of animals in LL, nor were any phase shifts observed in the enucleated animals that were restrained following an injection of vehicle (Figs 1 and 2). Representative activity records for animals in the various groups are shown in Figs 3 and 4.

The results of the present study raise important questions about the physiological mechanisms by which dark pulses induce phase shifts in the circadian clock of mammals and indicate that previous hypotheses regarding these mechanisms may be incorrect. The effects of dark pulses on circadian rhythmicity have been related to entrainment to the light-dark cycle and thought to involve light input pathways to the circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN)^{5,8,14-16}. Light-dark information is transmitted from ganglion cells in the retina to the biological clock in the SCN either directly via the retinohypothalamic tract or indirectly through the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) (refs 15 and 17). This latter pathway involves axonal projections from retinal ganglion cells to the IGL, and projections from the IGL to the SCN in which neuropeptide Y (NPY) appears to be the active neurotransmitter^{14,16,18,19}. The finding that both dark pulses and microinjections of NPY in the SCN region elicit similar phase shifts in the circadian rhythm of locomotor activity has led to the hypothesis that NPY may function as a neurotransmitter that communicates light-dark

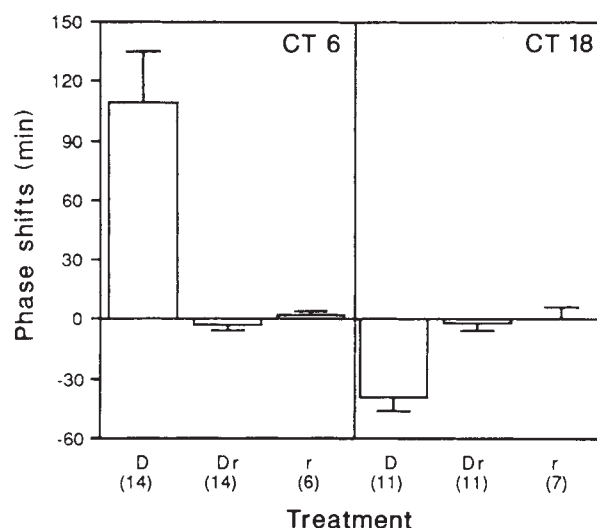


FIG. 1 Mean ($\pm$ s.e.m.) phase-shifts in the activity rhythm of hamsters maintained in constant light who were subjected, beginning at either CT 6 (left panel) or CT 18 (right panel), to a 6 h dark pulse (D) and/or a 6 h period of restraint (r). A value above the solid line indicates an advance in the onset of activity, a value below indicates a delay. The values within parentheses indicate the number of animals tested for each condition. The mean phase shift in the activity rhythm in response to the dark pulse was significantly greater ($P < 0.001$, one tail paired Student's *t*-test) in the unrestrained than in the restrained hamsters at both circadian times tested. METHODS. The effects of immobilization on phase shifts normally induced by pulses of darkness were assessed in 25 hamsters, 15-18 days after they were transferred to LL. The animals were exposed to a 6-h dark pulse beginning either 6 h before (CT 6; $N=14$) or 6 h after (CT 18; $N=11$) the onset of activity on two separate occasions separated by at least 12 days; exposure to dark pulses at these circadian times induce, respectively, phase advances or phase delays in the activity rhythm⁵. The animals were immobilized during one of the dark pulses and left unrestrained during the other, with approximately half the animals being either restrained or unrestrained during each of the dark pulses. A control age-matched group of 13 animals free-running in constant light for at least 23 days was restrained in the same room for a single 6 h period beginning at CT 6 ($N=6$) or CT 18 ($N=7$) in the absence of any change in the lighting environment. Immobilization was achieved by placing the animal in a closed transparent polypropylene tube (10 cm long $\times$ 5 cm diameter) provided with air vents for a period of 6 h. Phase shifts in the free-running activity rhythm were assessed as previously described^{6,9}.

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information from the IGL to the SCN^{14,16}. The present data, and recent findings of N. Mrosovsky and colleagues (personal communication) that confinement to a small nest box can also block the phase shifting effect of a dark pulse, suggest that phase shifts in response to dark pulses may actually be a secondary response to the increase in locomotor activity that is induced by such pulses. It is noteworthy that injections of NPY into the SCN region apparently also induce an increase in locomotor activity¹⁴, raising the possibility that phase shifts in response to NPY are not due to a direct action on SCN neurons involved in clock function, but are instead mediated by the induction of hyperactivity.

The results of the present study also raise important questions about the physiological mechanisms involved in benzodiazepine-induced phase shifts in the circadian clock. As benzodiazepines are thought to act by potentiating the action of the inhibitory neurotransmitter γ -aminobutyric acid (GABA), and as GABA is a major neurotransmitter within the SCN, it has been speculated that benzodiazepines may exert their effects on circadian rhythmicity by a direct action on SCN neurons²⁰. On the basis of pharmacological studies involving GABA antagonists and benzodiazepines in combination with light pulses, it has been suggested that the GABA-benzodiazepine receptor complex may be involved in transmitting light-dark information to the circadian clock^{21,22}. The recent demonstration that lesions of the LGN can block the phase shifts induced by triazolam²³ is compatible with an effect of benzodiazepines on the circadian clock via an action on photic inputs to the SCN. Rather than pointing to an effect of triazolam on the SCN or photic input pathways to the biological clock, the present findings indicate that the effects of triazolam on the clock are actually mediated by an increase in locomotor activity. The LGN may be involved in this response, as LGN lesions also seem to block the ability of triazolam to induce an acute increase in locomotor activity of hamsters²³. At the present time, neither the specific components of activity, nor the input pathways by which these components can alter circadian rhythmicity, has been identified.

The finding that immobilization can block phase shifts induced by dark pulses or triazolam seems to relate specifically to shifts associated with hyperactivity. Phase shifts in response to light pulses, which are not associated with an increase in locomotor activity, are not blocked by restraining the animal (O.V.R., unpublished results). Thus, it is unlikely that our restraint procedure is inducing some unspecific 'stress response' that is blocking the phase-shifting effects of either triazolam or the dark pulses. It should be noted that although the restraint procedure alone did not induce phase shifts in the circadian

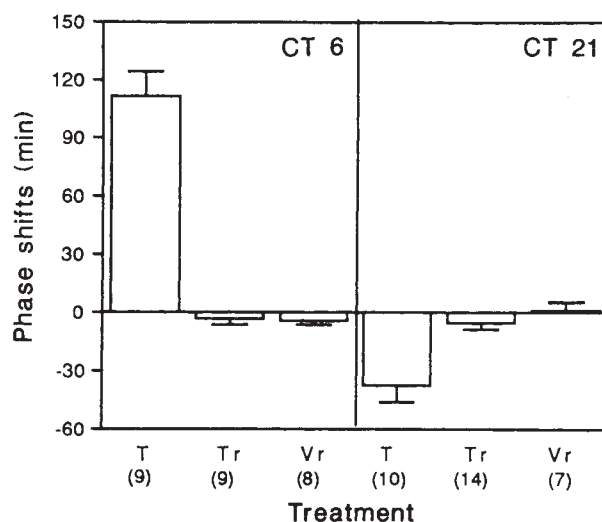
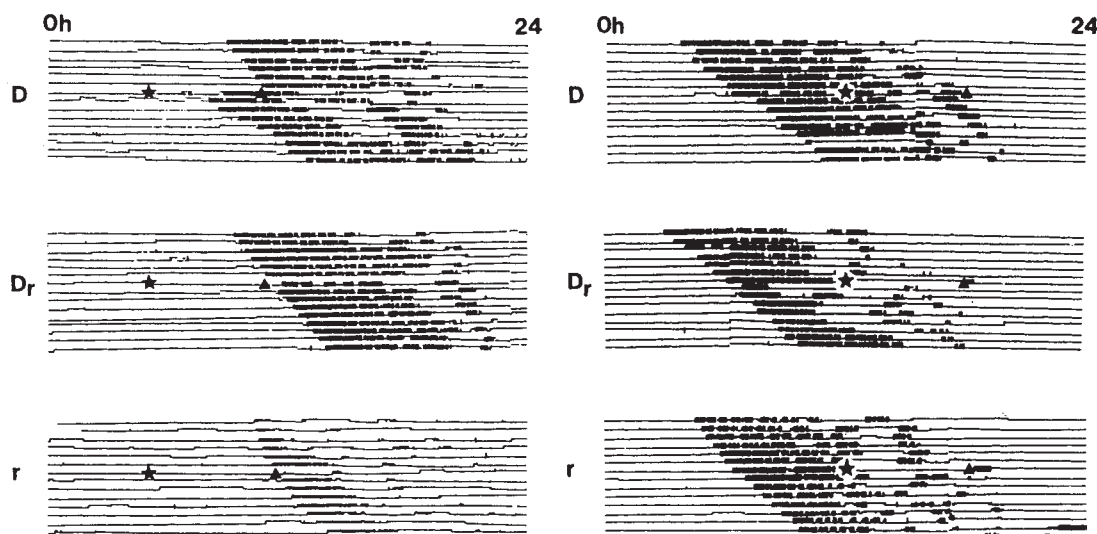


FIG. 2 Mean ($\pm$ s.e.m.) phase-shifts in the activity rhythm of free-running hamsters that received, either at CT 6 (left panel) or CT21 (right panel), a systemic injection of 2.5 mg triazolam (T) or vehicle (V). The animals were either left undisturbed or were restrained (r) for 6 h following the injections. A value above the solid line indicates an advance in the onset of the activity, a value below indicates a delay. The values within parentheses indicate the number of trials for each group of animals. The mean phase shift in the activity rhythm in response to the injection of triazolam was significantly greater (one tail unpaired Student's *t*-test) in unrestrained than in restrained hamsters, at CT6 ($P < 0.001$) or CT21 ($P < 0.01$).

METHODS. The effects of immobilization on phase shifts normally induced by injections of triazolam were assessed in 24 enucleated hamsters. The animals received intraperitoneal injections (0.1 ml) of either vehicle (dimethyl sulphoxide) or 2.5 mg triazolam (Halcion, Upjohn) dissolved in vehicle at either CT 6 or CT 21. These injection times were selected on the basis of previous studies demonstrating that administration of this dose of triazolam at CT 6 or CT 21 induces, respectively, the largest phase advance or phase delay in the activity rhythm of hamsters housed in constant darkness⁹. Individual hamsters received injections of vehicle or triazolam on 2-4 occasions, on days 12, 20, 29 and 49 after enucleation. No animal was subjected to the same experimental or control procedure more than once. See Fig. 1 for further details.

rhythm of activity in the present study, the animals were only restrained during their circadian day at a time when they are normally inactive (CT 6-12) or there is reduced activity (CT 18-24 or CT 21-03). Preliminary data indicate that restraint alone during the normally active period can by itself induce small phase shifts in the activity rhythm of hamsters. In view

FIG 3 Representative sections from the wheel-running activity records of six hamsters housed in constant light before and after they were subjected, beginning at either CT 6 (left panels) or CT 18 (right panels), to a 6-h dark pulse (D) and/or a 6-h period of restraint (r). The beginning and end of the dark pulse and/or the restraint procedure are represented by a star and triangle, respectively. Each horizontal line represents a single 24 h period and successive days are plotted from top to bottom. In each record, the solid bars represent periods of intense wheel-running.



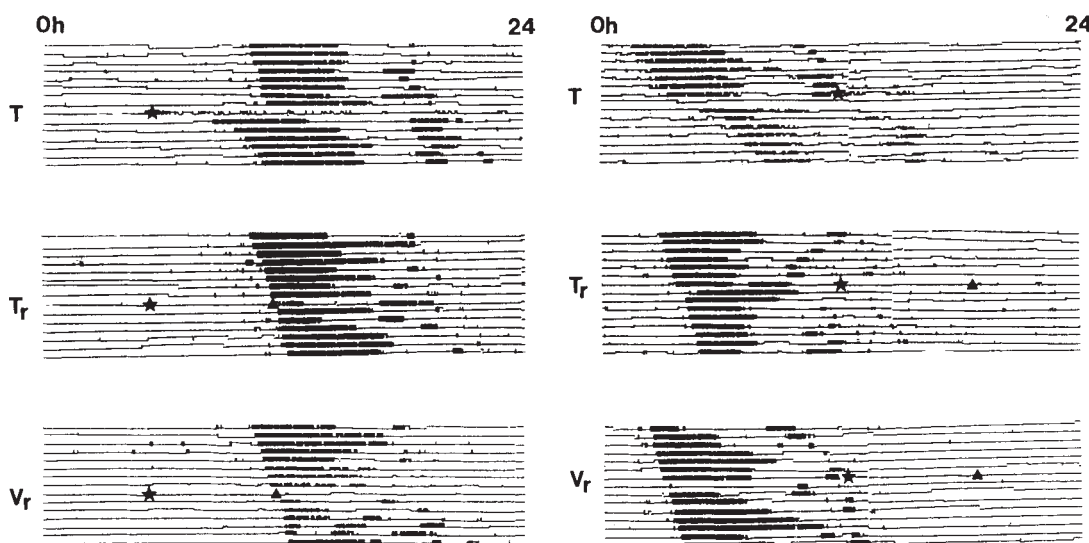


FIG 4 Representative sections from the wheel-running activity records of six free-running hamsters before and after they were injected with 2.5 mg triazolam (T) or vehicle (V) at either CT 6 (left panels) or CT 21 (right panels). Coincident with the injections, the animals were either left undisturbed (top panel in each row) or were restrained (r) for six hours. Exact time of the injection (and introduction to the restraining tube), star; Exact time of removal from the restraining tube, triangle.

of the possibility that a dark pulse or an injection of triazolam might induce an immediate phase shift in the circadian clock regulating the rhythm of locomotor activity (for example, an injection of triazolam at CT 6 might advance the clock immediately to CT 8), we have examined whether restraint alone between CT 8–14 would alter the activity rhythm. However, restraint during this time period did not induce a significant phase shift in the activity rhythm of free running hamsters (phase shift = -9 ± 5 min, $N = 7$). Thus, the finding that immobilization totally blocks phase shifts associated with stimulated physical activity seems to be due to the prevention of increased activity *per se*, and is not due to a restraint-induced phase shift in the opposite direction that cancels out the phase shift resulting from hyperactivity.

The finding that changes in the activity state of animals can have feedback effects on the circadian clock may have important clinical implications^{12,13}. A number of mental and physical disorders have been associated with abnormal circadian rhythms and circadian dysfunction has been implicated in some of the health problems associated with rapid movement across time zones and shift work^{24–27}. It is of particular interest to determine if the human circadian clock can be altered by changes in arousal state (either physical or mental). Such effects on human circadian rhythms might underlie some of the circadian abnormalities that have been found in humans, but do not appear to occur in other species, such as rhythms with periods longer than 30 h and the desynchronization of rhythms from each other²⁸. □

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ACKNOWLEDGEMENTS. This work was supported by a 'Science de la Vie' contract of the Ministère de la Politique Scientifique de Belgique, grants from the NIH and NIMH, the Upjohn Company and the Fond National de la Recherche Scientifique (O.V.R.). We are grateful to Mademoiselle Pierrette Denis for technical assistance.

Persistent and transcriptionally-dependent increase in protein phosphorylation in long-term facilitation of *Aplysia* sensory neurons

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FROM certain perspectives, short- and long-term memory seem to be a single behavioural process whose duration is a graded function of the number of training trials^{1–3}. Yet some clinical conditions can dissociate short- from long-term memory in human beings⁴, and inhibitors of protein or RNA synthesis can selectively block the long-term process in experimental animals^{5–7}. Studies of memory for sensitization in the gill- and siphon-withdrawal reflex in *Aplysia* indicate that both the behavioural similarities and the differences are reflected in intrinsic cellular mechanisms in the sensory and motor neurons participating in memory storage. Although the long-term change in the synaptic connection between the sensory and motor neurons resembles a graded extension of the short-term change, its induction is selectively blocked by inhibitors of transcription or translation⁸. We have now examined the molecular mechanisms in the sensory neurons that might account for the graded similarity between short- and long-term memory, as well as those that might contribute to the differential sensitivity to inhibitors of macromolecular synthesis. We find that a single exposure to 5-HT (a transmitter released in response to behavioural sensitizing stimuli) or cyclic AMP (a second messenger

Received 28 November 1988; accepted 20 March 1989.

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for 5-HT), which produce short-term facilitation between the sensory and motor neurons lasting minutes⁹⁻¹³, leads to a short-term phosphorylation of 17 substrate proteins that is not dependent on transcription or translation. Repeated or prolonged exposure to serotonin or cAMP, which induce long-term changes in synaptic transmission lasting one or more days^{9,11,12}, induce long-term changes in phosphorylation of the same 17 proteins that are now dependent for their induction on both translation and transcription. Thus, one of the functions of the genes and proteins required for long-term facilitation may be to maintain actively in the sensory neurons an increased phosphorylation of the same set of substrate proteins involved in eliciting the physiological effects of the short-term process.

To investigate biochemical mechanisms relating short- and long-term facilitation in the connections between the sensory and motor neurons in *Aplysia*, we first applied a single 2-min pulse of 5-HT (40 μ M) to sensory neurons that had been labelled with ³²P to isotopic equilibrium. A single pulse of 5-HT elicited a specific increase in phosphorylation of at least 17 proteins (Fig. 1 and Table 1). As Fig. 1 indicates, certain proteins appear as doublets and, although we have identified these as individual proteins, they may in fact be isoforms of the same protein. The proteins phosphorylated range in relative molecular mass from 15,000 to 56,000 (15K-56K) and in pI from 4.7 to 7.3. The alteration of a relatively large number of proteins suggests that even a single application of a modulatory transmitter important for learning, such as 5-HT, elicits changes in a wide variety of biochemical processes in sensory neurons (see refs 14 and 15). No proteins were found to have their phosphorylation decreased in response to 5-HT. This phosphorylation was transient; a single 2-min application of 5-HT did not lead to long-term (lasting 24 h) changes in protein phosphorylation. Moreover, the

increase in phosphorylation after a 2-min application of 5-HT was not affected by inhibitors of protein synthesis (Fig. 2 and data not shown). We do not as yet know the identity of most of these substrate proteins, but recent biochemical experiments indicate that one protein (number 12) is actin. Although physiological evidence suggests that the S-K⁺ channel (or a regulatory protein that acts on it) is also phosphorylated¹⁶, we are at present unable to determine which, if any, of these biochemically detected phosphoproteins is related to the S-channel.

The cAMP-dependent protein kinase mediates certain effects of 5-HT on sensory neurons¹⁷⁻²⁰ (for reviews see refs 26 and 33). We therefore examined how the response of sensory neurons to a single application of a cell-permeable cAMP analogue compared with the response to 5-HT. Combined application for 10 min of cAMP analogue (100 μ M chlorophenylthio cAMP) and a phosphodiesterase inhibitor (100 μ M of either isobutylmethylxanthine (IBMX) or RO20-1724) led to an increase in phosphorylation of the same 17 proteins that had their phosphorylation increased in response to 5-HT (Table 1). Moreover, for most proteins there was a similarity between 5-HT and cAMP in the degree of increase in the phosphorylation of specific proteins (notable exceptions being spots 6, 7 and 10). The differences observed might be due to permeability barriers or subcellular compartmentalization of the cAMP analogue, or selective activation of certain isotypes of cAMP-dependent protein kinase by one or the other treatment. These results provide direct and independent evidence that 5-HT leads to activation of the cAMP-dependent protein kinase in sensory neurons, and are consistent with earlier studies indicating an important role for the cAMP-dependent kinase in the response of sensory cells to 5-HT^{20,21}. Moreover, this overall pattern of phosphorylation was not observed with another cellular stimulus. Phorbol esters

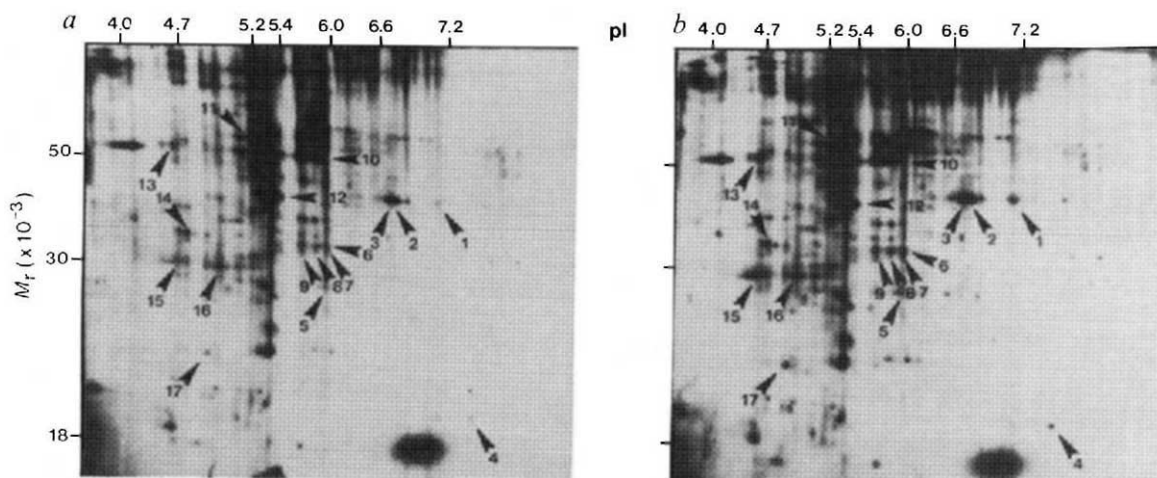


FIG. 1 Autoradiographs of 2-dimensional gels from ³²P-labelled *Aplysia* pleural sensory clusters. *a*, Control (unstimulated sample). *b*, 5-HT-treated (40 μ M 5-HT for 2 min). This experiment is representative of the six separate experiments for which averages are shown in Table 1. Protein spots whose ³²P-content reproducibly increased are marked with arrows (see Table 1). Spots listed in Table 1 are indicated in this figure, along with the corresponding number from the table. Because of overexposure or crowding in certain areas of the gels, certain spots are less clear at this exposure than others. These spots were quantitated using films exposed for shorter periods of time.

METHODS. *Aplysia californica* (100-200 g) pleural sensory neuron clusters were prepared as described previously³¹. These bilaterally symmetrical and homogeneous clusters, of approximately 200 sensory neurons each, were kept as pairs from one animal. Each pair was used for a single experimental determination, with one cluster serving as control and one as treated. This allowed us to perform intra-animal normalization and served to eliminate the inter-animal variability found for basal ³²P incorporation in *Aplysia* (see also ref. 31). Each cluster was labelled for 22-24 h with 20 μ Ci per 100 μ l ³²PO₄ in culture medium supplemented with amino acids, sugars, vitamins

and penicillin-streptomycin¹¹. In preliminary experiments (data not shown), we confirmed the results of Bernier *et al.*²¹ that this incubation period labels the *Aplysia* neuronal ATP pool to apparent isotopic equilibrium. In additional control experiments, we confirmed that the labelling of each protein found to change in response to 5-HT application was also labelled to apparent isotopic equilibrium. Thus, a change in ³²PO₄ content for a protein reflects an increase in phosphate content of that protein. Cells labelled using this protocol exhibited normal physiological characteristics, including spike broadening in response to 5-HT application (data not shown). After stimulation of the sensory cluster, the incubation was terminated by the addition of lysis buffer containing 0.3% SDS, 5% β -mercaptoethanol, 20 mM Tris, pH 8.0. The samples were then treated with DNase-RNase for 5 min on ice. Cell extracts were analysed by two-dimensional gel electrophoresis on each sample, using a pH 3-10 first-dimension, isoelectric-focusing gel and a 12.5% acrylamide second-dimension gel^{6,32}. Three autoradiographic exposures using enhancing screens were performed at -70 °C for periods of from 1 day to 3 weeks depending on the ³²P content of each sample. A typical intermediate-level exposure is shown above. (See refs 10 and 32 for a complete description of the computer-aided analysis used here.)

TABLE 1 Phosphorylation of selected proteins from *Aplysia* sensory neurons

Spot	$M_r \times 10^{-3}$ pI		2 min 5-HT assay = 2 min	10 min cAMP assay = 10 min	2 h 5-HT assay = 24 h	2 h cAMP assay = 24 h	2 h 5-HT plus anisomycin assay = 24 h	2 h 5-HT plus actinomycin D assay = 24 h	2 h cAMP plus anisomycin assay = 24 h	2 h cAMP plus actinomycin D assay = 24 h
			$\bar{x}$, $n=6$ s.e.m.	$\bar{x}$, $n=7$ s.e.m.	$\bar{x}$, $n=7$ s.e.m.	$\bar{x}$, $n=5$ s.e.m.	$\bar{x}$, $n=3$ s.e.m.	$\bar{x}$, $n=3$ s.e.m.	$\bar{x}$, $n=3$ s.e.m.	$\bar{x}$, $n=3$ s.e.m.
1	44.7	7.2	2.17 0.32	4.17 1.12	4.26 1.78	3.14 1.22	1.16 0.06	1.18 0.15	0.89 0.03	0.74 ($n=2$)
2	44.7	6.65	6.50 2.08	4.63 1.41	2.75 0.73	1.71 0.31	1.17 0.12	0.82 0.23	0.94 0.26	0.96 ($n=2$)
3	44.7	6.70	5.49 2.15	4.65 1.26	3.90 0.99	1.74 0.41	1.27 0.22	0.82 0.30	1.22 0.27	1.46 ($n=2$)
4	15.5	7.3	2.26 0.20	1.50 0.18	2.23 0.46	1.44 0.19	1.22 0.21	1.37 0.32	1.73 0.78	1.00 ($n=2$)
5	28	6.0	1.50 0.19	1.83 0.24	3.28 1.70	1.76 0.16	1.14 0.15	1.34 0.20	0.96 0.02	0.84 0.13
6	33.9	6.0	5.73 1.88	2.65 0.51	6.43 3.80	2.45 0.66	1.02 0.11	0.88 0.29	0.81 0.04	0.91 0.12
7	33.1	6.0	5.38 1.78	2.61 0.46	6.02 3.80	4.85 2.68	1.11 0.07	1.03 0.11	0.75 0.02	0.96 0.27
8	33.9	5.7	6.12 2.33	3.65 1.53	3.80 1.00	2.15 0.56	1.06 0.12	0.99 0.12	1.20 0.23	0.99 0.36
9	33.1	5.5	3.64 0.65	2.07 0.60	2.09 0.49	1.66 0.19	1.08 0.07	1.11 0.06	1.12 0.17	0.92 0.03
10	52.5	5.6	2.70 0.50	5.70 2.44	3.24 0.62	3.39 0.91	1.26 0.54	1.13 0.19	0.91 0.07	0.67 0.11
11	55.6	5.2	4.15 1.19	3.86 1.14	2.67 0.38	2.08 0.41	1.09 0.08	0.88 0.14	1.03 0.22	0.91 0.12
12	43	5.3	1.55 0.16	2.32 0.75	1.59 0.36	1.52 0.23	1.00 0.02	1.01 0.20	1.05 0.03	0.85 ($n=2$)
13	51.9	4.7	2.41 0.26	2.78 0.54	3.09 0.82	1.90 0.35	1.06 0.29	1.10 0.18	1.21 0.07	0.92 0.11
14	36.3	4.75	1.70 0.23	1.43 0.14	2.49 0.54	1.49 0.24	1.11 0.01	0.92 0.09	0.98 0.09	0.99 0.07
15	30.6	4.7	4.48 1.47	5.83 2.54	4.00 1.52	1.66 0.25	0.99 0.19	0.95 0.36	1.49 0.13	1.18 0.38
16	30.9	5.0	1.81 0.09	1.51 0.14	2.34 0.33	1.67 0.24	0.99 0.10	1.06 0.13	1.00 0.13	0.78 0.04
17	20.9	4.95	2.57 0.41	2.45 0.26	2.43 0.41	3.21 1.33	0.87 0.20	1.11 0.17	1.25 0.23	0.77 0.17

Effects of various treatments on the phosphorylation of 17 selected proteins from *Aplysia* sensory neurons. Results shown are the means ($\pm$ s.e.m.) of the ratios of stimulated/control, for the indicated number of experiments. Of ~ 300 –400 phosphoprotein spots per gel, only these 17 were found to change reproducibly as a result of the indicated treatments. Quantitative information for the ^{32}P content of control and stimulated samples was determined by densitometric scanning. Spots were matched and ratios (stimulated/control) were calculated using the PDQuest computer analysis system. Treatments were 40 μM 5-HT for either 2 min or 2 h, or 100 μM 6-(4-chlorophenylthio)-cyclic AMP plus 100 μM phosphodiesterase inhibitor (either isobutylmethylxanthine or RO20-1724) for either 10 min or 2 h. Where anisomycin (10 μM) or actinomycin D (50 $\mu\text{g ml}^{-1}$) were used, they were added at least 30 min before addition of the stimulus. Effects on protein phosphorylation were assayed either at the end of the stimulus period or 24 h after washout of all stimuli and inhibitors. In additional control experiments using ^{35}S -methionine labelling, we observed that none of these 17 proteins exhibited an increased ^{35}S -labelling 24 h after prolonged stimulation with 5-HT. Thus, the increased ^{32}P -labelling observed does not reflect an increase in amount of protein present, but rather its ^{32}P content.

that activate protein kinase C elicit a different pattern of phosphorylation. As at least two substrate proteins are common to both cAMP and phorbol esters (data not shown), however, and as 5-HT is known to translocate several protein kinases in sensory neurons^{22–24} these data do not exclude recruitment by 5-HT of other second messenger systems. In addition, we cannot exclude the possibility of phosphorylation events occurring below the limit of detection of this assay system.

Five repeated applications of or prolonged exposure to 5-HT lead to long-term changes in the synaptic strength of *Aplysia* sensory neurons that persist for at least 24 h following removal of the 5-HT^{9,11,25}. The biophysical parameters that characterize this long-term change are strikingly similar to those that are observed following short-term facilitation. For example, a single application of 5-HT leads to an increase in transmitter release accompanied by an increase in excitability (due to depression of S-K current) that is rapid in onset and reversible within minutes^{9,11,25}. Repeated or prolonged application of 5-HT leads to a similar enhanced transmitter release and increased excitability, but now these changes persist for at least 24 h after the washout of 5-HT^{9,12}. How is this change maintained in the long-term? Can repeated exposures to 5-HT lead to a persistence in phosphorylation that might mediate the persistent functional effects?

To examine these questions, we gave five pulses of 5-HT to sensory clusters (or simply exposed them to 5-HT for 2 h) and assayed for effects on protein phosphorylation 24 h later. As illustrated in Table 1, repeated or prolonged application of 5-HT caused a long-term effect on protein phosphorylation in sensory neurons. Particularly striking is the finding that the same 17 proteins, whose phosphorylation was increased only transiently after a 2-min application of 5-HT, now had their phosphorylation increased when assayed at 24 h after removal of the 5-HT. Similar results were obtained following a 2-h application of a cAMP analogue (plus isobutylmethylxanthine or RO20-1724). The cAMP analogue also caused a selective increase in the phosphorylation of the same subset of 17 proteins and again the phosphorylation persisted (Table 1). Thus, as is the case with 5-HT, cAMP caused qualitatively similar short- and long-term effects. In general, however, the cAMP analogue gave a less robust increase in protein phosphorylation than 5-HT, as measured at 24 h (Table 1). One possible explanation for this

difference could be that chlorophenylthio-cAMP could not adequately maintain, for 2 h, an elevated level in relevant parts of the cell, perhaps due to chemical breakdown or limited permeability. An alternative possibility is that 5-HT recruits additional mechanisms for induction of long-term effects. The data nonetheless suggest that elevation of cAMP contributes importantly to the mechanism whereby 5-HT induces the persistent phosphorylation effect.

To gain insight into possible molecular mechanisms underlying induction of the persistent effect on protein phosphorylation by 5-HT and cAMP, we examined the consequences of inhibiting translation and transcription during stimulation of the cells. Anisomycin, an inhibitor of translation, and actinomycin D, an inhibitor of transcription, have been shown previously to block the long-term biophysical effects of 5-HT in sensory cells^{9,11}. As shown in Figs 2 and 3 and Table 1, application of anisomycin during 5-HT treatment blocked the acquisition of the persistent phosphorylation effect. This finding suggests that induction of the persistent phosphorylation effect requires the synthesis of new proteins in the period during which 5-HT is applied. Induction of the persistent phosphorylation effect also appears to require active transcription. Application of actinomycin D during 5-HT or cAMP stimulation blocked the induction of the persistent phosphorylation effect (Fig. 2 and Table 1). By contrast, transcriptional or translational inhibitors did not block the transient increase in phosphorylation caused by a single, brief application of 5-HT or cAMP (Fig. 2 and data not shown). These actions on the pattern of phosphorylation are strikingly similar, phenomenologically, to the action of anisomycin and actinomycin D on induction of long-term synaptic facilitation of the sensory neuron. Thus, the two long-term changes, the facilitation of transmitter release and the induction of the persistent phosphorylation effect, share a common dependence on protein and RNA synthesis for their induction.

That the persistent effects on protein phosphorylation in the sensory neurons require protein and RNA synthesis suggests that changes in transcription, perhaps involving expression of new genes, might be needed. This possibility raises the question: how might a change in gene expression lead to a persistent increase in phosphorylation of the substrates for the cAMP-dependent protein kinase in the cell? One possibility is that the expression of genes for the regulatory subunits of cAMP-depen-

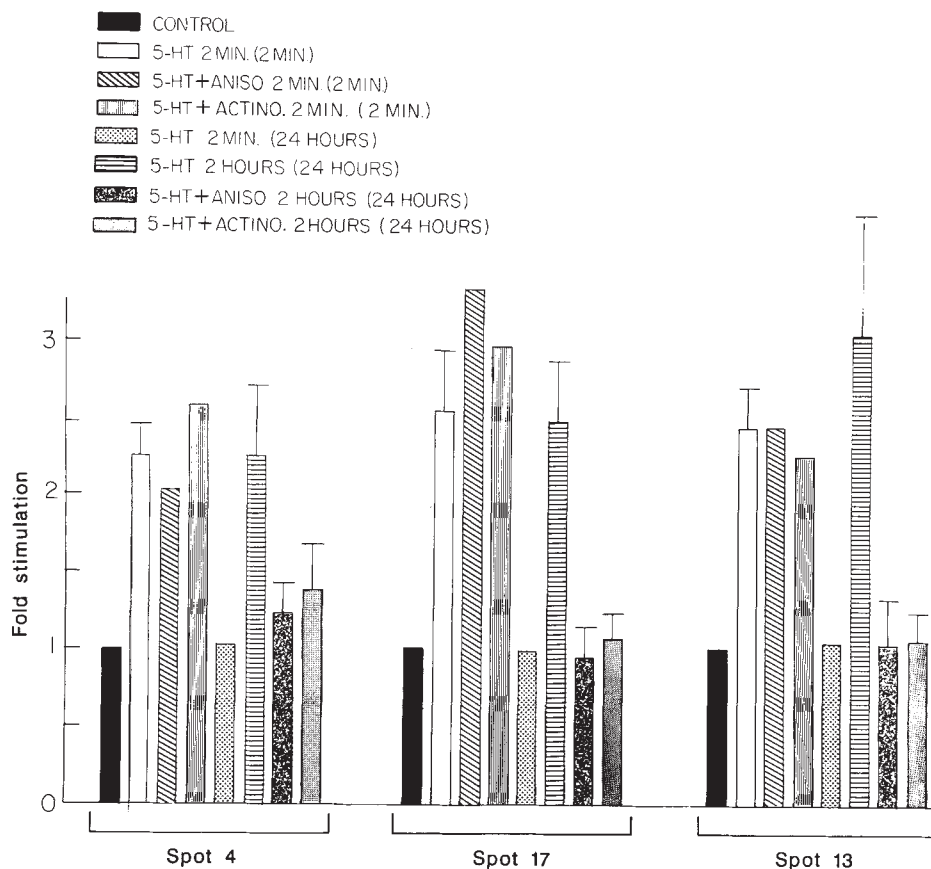


FIG. 2 Short- and long-term effects of 5-HT on the phosphorylation of individual proteins. Values given are the ratios of stimulated to control for ^{32}P -content of three representative spots, and error bars are s.e.m. Treatments were as follows: 5-HT 2 min (2 min), 40 μM 5-HT applied for 2 min and assayed at 2 min ($n=6$); 5-HT + ANISO 2 min (2 min), 40 μM 5-HT applied for 2 min in the presence of 10 μM anisomycin and assayed at 2 min ($n=1$); 5-HT + ACTINO 2 min (2 min), 40 μM 5-HT applied for 2 min in the presence of 50 $\mu\text{g ml}^{-1}$ actinomycin D and assayed at 2 min ($n=1$); 5-HT 2 min (24 h), 40 μM 5-HT applied for 2 min, washed out, and protein phosphorylation assayed at 24 h ($n=3$); 5-HT 2 h (24 h), 40 μM 5-HT applied for 2 h, washed out, and protein phosphorylation assayed at 24 h ($n=7$); 5-HT + ANISO 2 h (24 h), 40 μM 5-HT applied for 2 h in the presence of 10 μM anisomycin, then both agents washed out and protein phosphorylation assayed at 24 h ($n=3$); 5-HT + ACTINO 2 h (24 h), 40 μM 5-HT applied for 2 h in the presence of 50 $\mu\text{g ml}^{-1}$ actinomycin D, then both agents washed out and protein phosphorylation assayed 24 h ($n=3$). Where anisomycin or actinomycin D were applied, they were added either 30 min before stimulation (for samples incubated 2 h with stimulus) or 2.5 h before stimulation (for samples incubated for 2 min with stimulus) and present during stimulation.

dent protein kinase might be decreased (for an early discussion of this idea, see ref. 26). Alternatively, a protease could be induced that selectively degrades the regulatory subunits²⁷, or a new type of cAMP-dependent catalytic subunit might be induced that is less susceptible to inhibition by the regulatory subunits (see, for example, refs 26 and 28). Finally, there could be increased expression of a phosphatase inhibitor, or decreased expression of a phosphatase, in the sensory neuron.

The finding that the same substrate proteins whose phosphorylation is transiently increased upon a single application have their phosphorylation increased in the long-term following repeated application of 5-HT, might provide a molecular explanation for the apparently graded transition from short-term to long-term facilitation. In a larger sense, this mechanism could explain, at the cellular level, why aspects of the short- and long-term effects are indistinguishable except for duration. As is the case for short- and long-term behavioural memory and transmitter release by sensory neurons, however, the observation that inhibitors of transcription or translation can selectively block induction of the long-term effect demonstrates that even though the short- and long-term phosphorylation events are similar in final form, they must nonetheless be maintained by separable and distinct molecular processes.

As the memory for a variety of different learning processes in vertebrates as well as invertebrates seems similarly graded, a transcriptionally-dependent persistent increase in protein phosphorylation might prove to be a general mechanism for long-term memory. Indeed, a requirement for protein synthesis has recently been found in studies of long-term potentiation in the hippocampus (thought to use either protein kinase C or the Ca^{2+} -calmodulin dependent kinase) and in the long-term biophysical changes in the eye of *Hermisenda* (thought to use protein kinase C), both cellular correlates of long-term memory-type processes^{29,30}. This raises the interesting possibility that, as the long-term memory processes in different learning systems persist, they do so because the relevant protein kinases—such

as the cAMP-dependent kinase, protein kinase C, and the Ca^{2+} -calmodulin dependent kinase—might all be capable of a transcriptionally-dependent, persistent increase in activity. □

Received 12 January; accepted 23 March 1989.

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ACKNOWLEDGEMENTS. We thank Kathrin Hilten and Louise Katz for preparing the figures and Andrew Krawetz and Harriet Ayers for typing the manuscript. We also thank Drs M. Knapp, J.-F. Brunet, P. Pfaffinger and J. H. Schwartz for comments on an earlier draft of the manuscript.

An autosomal transcript in skeletal muscle with homology to dystrophin

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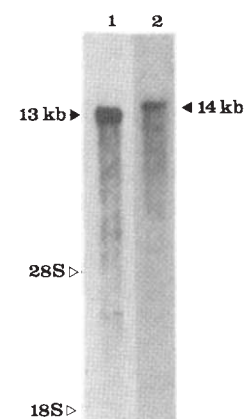
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THE Duchenne muscular dystrophy (DMD) gene has been localized to chromosome Xp21¹⁻⁶ and codes for a 14-kilobase (kb) transcript⁷ and a protein called dystrophin⁸, of relative molecular mass 427,000. Dystrophin is associated with the cytoplasmic face of muscle fibre membranes and its C-terminal domain is thought to mediate membrane attachment⁹⁻¹³. Although N-terminal and central domain structures share common features with other cytoskeletal components, no significant sequence similarity between the C-terminal region of dystrophin and other previously characterized proteins has been described. Here we report that fragments from the C-terminal domain of the DMD complementary DNA detect a closely related sequence which exhibits nucleic-acid and predicted amino-acid identities with dystrophin of approximately 65 and 80%, respectively. The dystrophin-related sequence identifies a 13-kb transcript in human fetal muscle and maps to chromosome 6. Thus, dystrophin may be a member of a family of functionally related large structural proteins in muscle.

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FIG. 2 Northern blot analysis of DMD cDNA and cDNA B3. Lane 1, Hybridization of 1.6-kb *EcoRI*-*HindIII* fragment of B3 to 5 µg mRNA isolated from cultured human fetal muscle cells¹⁸. The open arrow heads indicate the location of 28S and 18S RNAs. Lane 2, Hybridization of 1.4-kb fragment of Cf77a (Fig. 1) to northern blot of same gel as in lane 1.

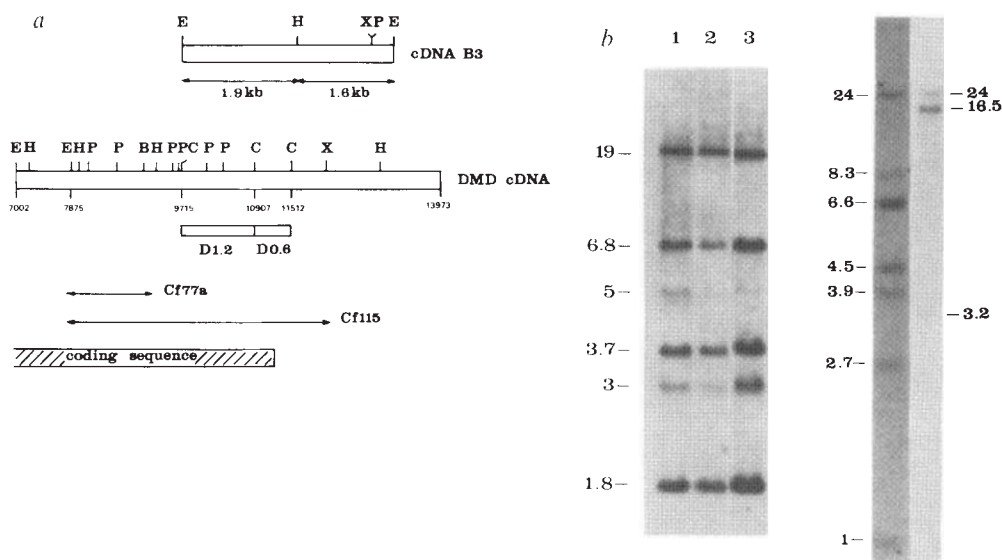
METHODS. The isolation of mRNA, electrophoresis and transfer to Gene-screen membrane (NEN) have been described¹⁹. Filters were probed with ³²P-labelled cDNAs at 10⁶ c.p.m. per ml hybridization solution. Hybridizations were performed at 42 °C in 5 × SSPE, 5 × Denhardt's solution and 50% (v/v) formamide, and washed at 50 °C for 30 min in 0.2 × SSC, 0.1% SDS. The probed filters were exposed to Fuji RX film (lane 1) and Kodak X-OMAT AR film (lane 2) for 16 h and 3 days, respectively, at -70 °C with intensifying screens. The membrane was stripped of probe between hybridizations by washing in 50% (v/v) formamide, 1 × SSC for 30 min at 75 °C then rinsing in 1 × SSC at 75 °C.



To isolate the full coding sequence of the dystrophin gene, cDNA clones were used to walk sequentially in an oligo(dT)-primed, fetal-muscle cDNA library¹⁴⁻¹⁵. When the library was screened with two contiguous *HindIII* DMD cDNA fragments (D1.2 and D0.6, Fig. 1) that encode the final C-terminal domain of dystrophin, two plaques were identified that defined distinct non-overlapping cDNAs. One cDNA was contiguous with the dystrophin sequence that we had already isolated. The restriction map of the other cDNA clone, designated B3, however, was different from that of the C-terminal region of the DMD cDNA (Fig. 1a). The 1.9-kb *EcoRI*-*HindIII* fragment of B3 hybridized to the DMD cDNAs D1.2 and D0.6, and this was the only region of similarity between cDNA B3 and the DMD transcript. The hybridization pattern of B3 to DNA from patients with large deletions in Xp21 was indistinguishable from that of a normal individual (Fig. 1b).

The 3.5-kb *EcoRI* fragment of B3 hybridizes to six different genomic *PstI* fragments which sum to ~39 kb (Fig. 1b); the

FIG. 1 Restriction maps and genomic analyses of cDNAs. **a**, The schematic restriction maps of cDNA B3 and the C-terminal region of the DMD cDNA indicate sites for *EcoRI* (E), *HindIII* (C), *HindIII* (H), *PstI* (P) and *XbaI* (X). The values below the DMD cDNA map indicate nucleotide number relative to the transcription start site¹³. The open boxes represent *HindIII* cDNA fragments used as hybridization probes. The location and extent of the human fetal muscle cDNAs Cf77a and Cf115 are indicated by arrows¹⁵. The hatched box indicates the extent of the dystrophin coding sequence. **b**, Hybridization of the 3.5-kb *EcoRI* fragment of cDNA B3 to *PstI*-digested DNA samples (5.0 µg per lane) from patients 1290 (M.J. with a deletion of the entire dystrophin gene, lane 1) and 346 (J.S. with a deletion of 5 kb of dystrophin coding sequence at the 3' end, lane 2)¹⁷ and a male control (lane 3). The sizes of the hybridizing fragments are indicated on the left in kilobase pairs. **c**, Hybridization of 1.2-kb (D1.2) and 0.6-kb (D0.6) *HindIII* fragments from the C-terminal region of the dystrophin transcript to *PstI*-digested DNA from a female. The Southern blot was hybridized with D1.2, then this probe was removed and the blot was hybridized with D0.6.



METHODS. DNA was transferred to Hybond N (Amersham) after electrophoresis and hybridized in 50% formamide, 3 × SSC at 42 °C. The blots were washed at 65 °C for 30 min in 3 × SSC, 0.1% SDS and exposed to Fuji RX film at -70 °C for 3-5 days with intensifying screens.

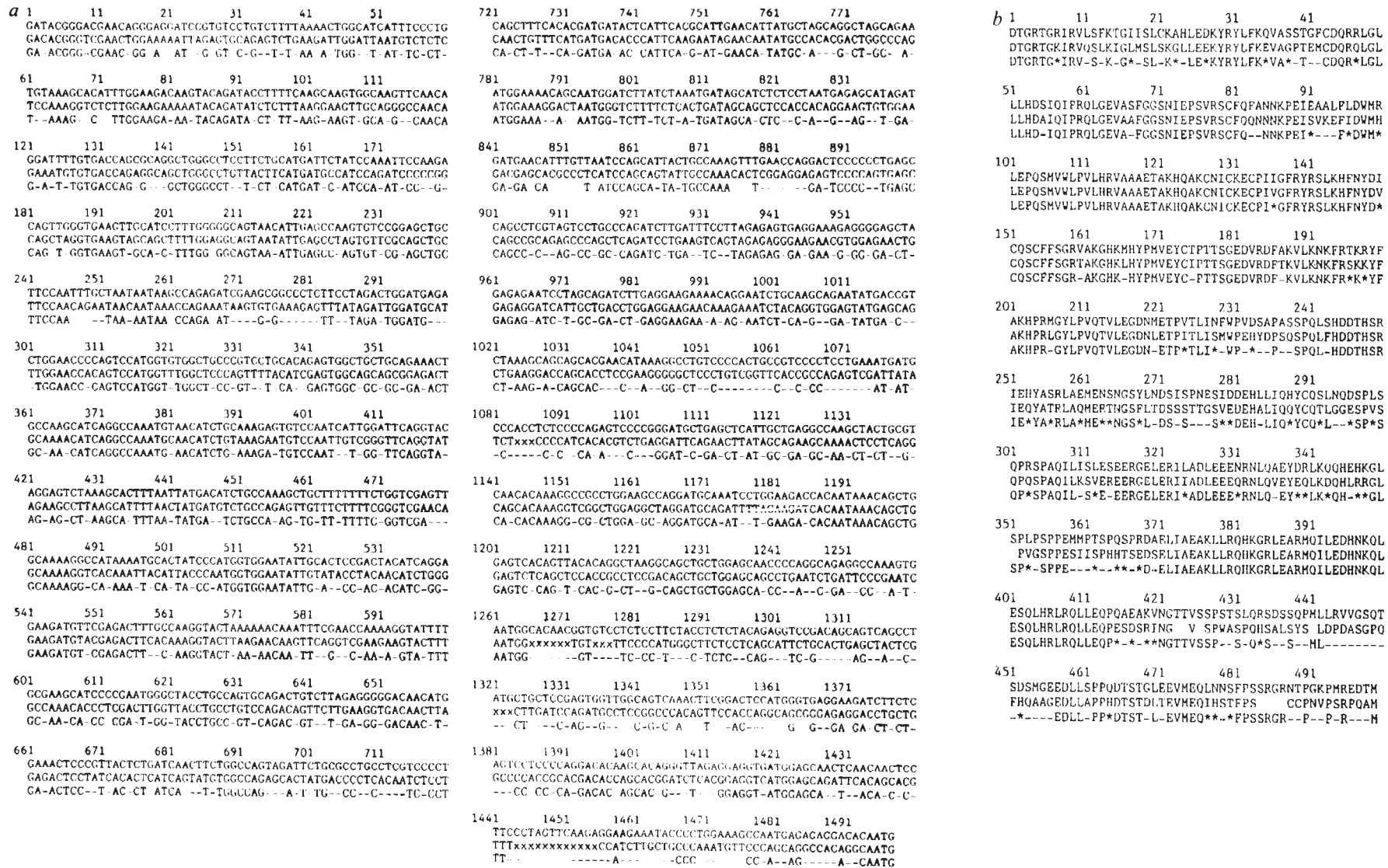


FIG. 3 Sequence comparison of 1.5 kb of B3 with the C-terminal region of the DMD transcript and protein product. *a*, The nucleotide sequence of the DMD transcript (top)¹³ is aligned with that of B3 (middle) on the basis of perfect matches. Bottom row, consensus sequence. Dashed line, no consensus. DNA sequencing was performed by the dideoxy chain-termination method²⁰ using the random sheared procedure²¹. DNA sequences were assembled and analysed using the computer programmes VTUTIN²² and HOMED²³. The values above the sequences indicate nucleotide number. Nucleotide number 1 corresponds to position 9,767 for the DMD transcript sequence.

and to position 24 from the *Eco*RI site of the 1.9-kb fragment of B3 (Fig. 1). *b*, The amino-acid sequence of dystrophin (top) and that deduced from the nucleotide sequence of B3 (middle) indicated in panel *a*, are aligned on the basis of perfect matches. Bottom row, consensus sequence. An asterisk indicates conservative amino-acid replacements. The values above the sequences indicate amino-acid number. Numbers 1 and 499 correspond to amino acids 3,187 and 3,685 (translation end) of the dystrophin protein sequence, respectively.

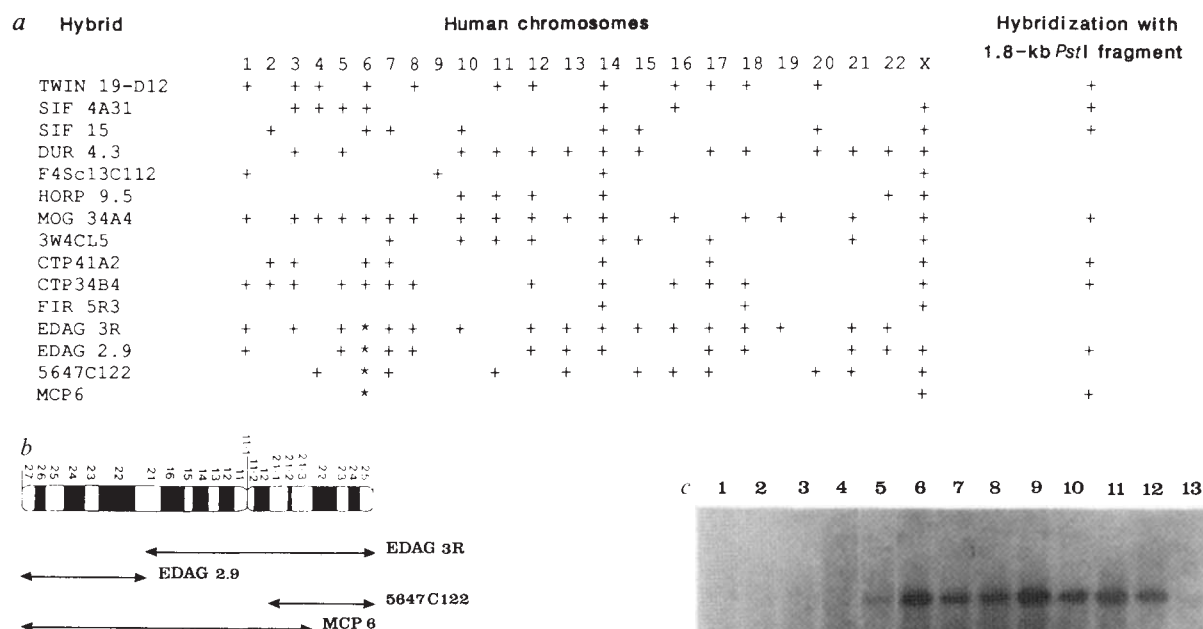


FIG. 4 Mapping of 1.6-kb *EcoRI*-*HindIII* fragment of B3 to human chromosomes. **a**, The human chromosomes in the human-rodent somatic cell hybrids have been described previously²⁴⁻²⁷. An asterisk indicates a translocated chromosome. An example of the data from which this figure is constructed is shown in panel **c**. **b**, Schematic representation of chromosome 6 fragments in somatic cell hybrids containing a translocated chromosome. The extent of chromosome 6 present in the hybrid cell lines is indicated by arrows. **c**, Southern blot analysis of DNA (~10 µg digested with *PstI* per lane): hamster (lane 1); hamster hybrid TWIN 19/D12 (lane 2); rat hybrid SIF 4A31 (lane 3); rat hybrid SIF 15 (lane 4); mouse hybrid DUR 4.3 (lane 5); mouse (lane 6); mouse hybrid F4Sc13C112 (lane 7); mouse hybrid HORP 9.5 (lane 8); mouse hybrid MOG 34A4 (lane 9); mouse hybrid 3W4CL5 (lane 10); mouse hybrid CTP41A2 (lane 11); mouse hybrid CTP34B4 (lane 12); human (lane 13). The hybridization and washing conditions were the same as those described in the legend to Fig. 1. The arrow indicates the location of the 1.8-kb, human-specific *PstI* fragment.

1.9-kb *EcoRI*-*HindIII* fragment hybridizes to all six *PstI* fragments, whereas the 1.6-kb *EcoRI*-*HindIII* fragment hybridizes to only one detectable genomic *PstI* fragment of 1.8-kb. The different hybridization profiles observed with the two fragments of B3 compare with that obtained for the C-terminal region of the DMD transcript. DMD probes D1.2 and D0.6 identify nine different genomic *PstI* fragments which sum to ~70 kb (Fig. 1c). The contiguous 2.5-kb of 3'-untranslated sequence of the DMD messenger RNA, however, seems to be encoded by only two exons⁷. A correlation of the hybridization profiles suggested that the 1.9-kb B3 fragment contains coding sequence, whereas the 1.6-kb B3 fragment may be non-coding.

The 1.6-kb *EcoRI*-*HindIII* fragment of B3 was hybridized to a northern blot of mRNA isolated from cultured human fetal muscle cells (myoblasts and myotubes). This probe does not cross-hybridize with the dystrophin cDNA. The fragment hybridized to a transcript of ~13 kb (Fig. 2, lane 1) that was clearly distinct from that of dystrophin, which was located at a slightly higher position in the gel (Fig. 2, lane 2). The B3 transcript is also present in adult skeletal muscle and gut (smooth muscle; unpublished observation).

The nucleotide sequence of ~3 kb of B3 was determined and compared with the entire sequence of the DMD transcript. The sequence of 1.5 kb of B3, localized to the 1.9-kb *EcoRI*-*HindIII* fragment, shows 65% identity with the nucleotide sequence of the coding region of D1.2 and D0.6 (Fig. 3a). An open reading frame (ORF) was identified in this region of similarity between the dystrophin and B3 cDNAs. The deduced sequence of 490 amino acids shows 73% identity with the C-terminal domain of

dystrophin (Fig. 3b); this similarity increases to 83% if conservative amino-acid substitutions are taken into consideration. The point of divergence between B3 and dystrophin DNA sequences is at the end of the ORF of both sequences (data not shown). The start site of the ORF in the 1.9-kb *EcoRI*-*HindIII* fragment of B3 begins 24 bases downstream of the *EcoRI* site. This 24-base pair (bp) sequence may be due to a cloning artefact because the 3.5-kb B3 cDNA has stop codons in all other reading frames and is clearly expressed. The isolation of more cDNAs that overlap the 1.9-kb fragment should enable us to extend the ORF and determine the full extent of the genomic locus encoding the 13-kb transcript.

The chromosomal localization of B3 was determined by hybridization to a panel of human-rodent somatic cell hybrid DNAs (Fig. 4). The sequence was localized to 6q21-qter by the presence of the human-specific 1.8-kb *PstI* fragment identified by the 1.6-kb *EcoRI*-*HindIII* fragment of B3. This cDNA fragment also hybridized to 15-kb and 7-kb *PstI* fragments in mouse, and rat and hamster, respectively. Homologous genomic fragments were observed in chicken and horse DNA (unpublished observation). The conservation of the predicted untranslated region of B3 across species has also been reported for the 3'-untranslated sequence of the dystrophin transcript¹⁶.

In summary, we have identified a large evolutionarily conserved transcript in human fetal muscle which is closely related to a region of dystrophin that does not show any homology to previously reported proteins. Thus dystrophin may be a member of a family of functionally related large structural proteins in skeletal muscle. □

Received 7 March; accepted 30 March 1989.

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ACKNOWLEDGEMENTS. We thank Jenny Bloomfield, Giles Reed, Lesley Rooke and Malcolm Hawkins for their research assistance and Helen Blaber for typing the manuscript. We also thank Michael Murphy for help in isolating cDNA B3. This work was supported by the Muscular Dystrophy Group of Great Britain, Wellcome Trust, the Medical Research Councils of Great Britain and New Zealand and the Muscular Dystrophy Association, USA.

Induction of angiogenesis during the transition from hyperplasia to neoplasia

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It is now well established that unrestricted growth of tumours is dependent upon angiogenesis^{1,2}. Previous studies on tumour growth, however, have not revealed when or how the transition to an angiogenic state occurs during early tumour development. The advent of transgenic mice carrying oncogenes that reproducibly elicit tumours of specific cell types³⁻⁶ is providing a new format for studying multi-step tumorigenesis^{7,8}. In one of these models, transgenic mice expressing an oncogene in the β -cells of the pancreatic islets heritably recapitulate a progression from normality to hyperplasia to neoplasia⁶. We report here that angiogenic activity first appears in a subset of hyperplastic islets before the onset of tumour formation. A novel *in vitro* assay confirms that hyperplasia *per se* does not obligate angiogenesis. Rather, a few hyperplastic islets become angiogenic *in vitro* at a time when such islets are neovascularized *in vivo* and at a frequency that correlates closely with subsequent tumour incidence. These findings suggest that induction of angiogenesis is an important step in carcinogenesis.

The insulin-producing β -cells are localized in approximately 400 focal endocrine islets embedded in the exocrine pancreas (Fig. 1a(i)). The neoplastic transformation of these cells has been accomplished through the use of a hybrid oncogene (*RIP-Tag*) that employs the rat insulin gene regulatory region to control expression of the SV40 large T oncoprotein⁶. We have studied one line of transgenic mice (*RIP1-Tag2*) in which virtually every β -cell already expresses the oncogene at birth⁹.

Hyperplasia of β -cells first becomes apparent at 4-6 weeks of age, and by 9.5 weeks, 50% of the islets are composed of proliferating β -cells¹⁰. Yet only a few islets progress to histologically distinct carcinomas, which are present by 12 weeks of age in all mice of this lineage. Thus, it seems that oncogene activation and consequent β -cell hyperplasia are necessary, but not sufficient, to produce a carcinoma.

To determine when neovascularization was initiated during β -cell tumorigenesis, immunohistochemical studies were performed using anti-laminin antibodies. Laminin is a ubiquitous basement membrane component that serves as an excellent marker for developing capillaries within normal¹¹ and neoplastic¹² tissues. Normal pancreatic islets and early hyperplastic islets contain tubular capillaries that appear homogeneous in form (Fig. 1a(i)). A subset of the hyperplastic islets, however, exhibit capillaries of varying shape and size as well as altered basement-membrane-staining patterns (Fig. 1a(ii)) due to formation of new capillary sprouts (Fig. 1b). Similar analyses of solid tumours revealed many new capillaries as well as complete disorganization of islet architecture (Fig. 1a(iii)).

The onset of new capillary growth within transgenic islets was confirmed using ³H-thymidine autoradiography. Normal islet capillaries did not incorporate thymidine during the labelling periods. This is consistent with previous reports of a very low ³H-thymidine labelling index of 0.01% in the capillaries of the pancreas¹³. By contrast, endothelial cells that incorporated ³H-thymidine could be detected within capillaries in a subset of hyperplastic islets and within every large carcinoma (Fig. 1c).

These histological analyses demonstrated that neovascularization is a quality of every solid tumour, but only of an occasional hyperplastic islet. The results imply that an angiogenic capability develops as a consequence of some localized change within the population of hyperplastic islets during the preneoplastic period. There are several alternative explanations: (1) Every hyperplastic islet composed of proliferating cells expressing the oncogene is angiogenic, but the surrounding environment of normal pancreas suppresses this intrinsic capability, a suppression from which an islet can sporadically escape; (2) when hyperplastic islets exceed a threshold size, angiogenesis is automatically elicited; or (3) there is an infrequent, positive switch to an angiogenic state by individual hyperplastic islets which is independent of size or surrounding environment.

To distinguish between these possibilities, we devised a novel *in vitro* assay to measure the response of capillary endothelial cells to individual hyperplastic nodules. Islets were removed from the pancreas by infusion of collagenase retrograde into the pancreatic ducts¹⁴ and then purified on a Ficoll gradient. Neither normal nor hyperplastic islets were larger than 400 μ m in diameter (data not shown). Therefore, a sieve of 560- μ m mesh size was employed to separate hyperplastic islets from

TABLE 1 *In vitro* angiogenesis assays

Age (weeks)	Angiogenic islets/total no. islets analysed			No. solid tumours per mouse				
4	0/40	0/40	0/358*	0	0	0	0	0
5	0/66			0				
6	0/138	0/78	0/79	1	0	1		
7	1/101			0				
8	1/100			2				
9	5/132	3/118	3/40	4	7	8		
10	2/100	0/97		4	9			
11	3/88			3				
12	5/98	4/118	2/83	1/30	4	7	6	5

Results of *in vitro* angiogenesis assays using islets isolated from 21 transgenic mice. Each ratio (angiogenic islets/number of islets) represents data from an individual mouse except where indicated. The number of solid tumours per mouse represents tumours retained by the 560- μ m mesh during isolation of islets from a single pancreas.

* Combined data from 3 mice.

tumours. Tumours were then retrieved from the sieve and analysed separately. Individual islets or tumours were placed into the wells of 24-well plates, each of which contained capillary endothelial cells ($70,000 \text{ ml}^{-1}$) in a solution of fibrillar collagen, which gels at 37°C (Fig. 2). The behaviour of each islet and its surrounding capillary endothelial cells was observed over a 1–2 week period.

During the first day of incubation, endothelial cells anchored in the collagen and elongated in a random orientation, but did not proliferate or form capillary sprouts. In the presence of islets isolated from normal adult mice, endothelial cells remained in a random orientation with little or no locomotion or proliferation (Fig. 3a). By contrast, every solid tumour induced a dramatic radial alignment of endothelial cells within 24 hours in a pattern that converged toward the implanted tumour (Fig. 3b). This assay thus distinguishes the normal condition from the tumour that arises out of it, based on identification of angiogenic activity *in vitro*.

With the specificity of the assay established, islets were analysed from RIP1-Tag2 mice at weekly intervals (from 4–12 weeks of age). None of the islets from 4–5-week-old mice stimulated the capillary endothelial cells. A small subset of the islets isolated from older transgenic mice, however, exhibited clear angiogenic activity (Fig. 3c–f). The response of the endothelial cells to hyperplastic islets or to tumours recapitulated the known stages

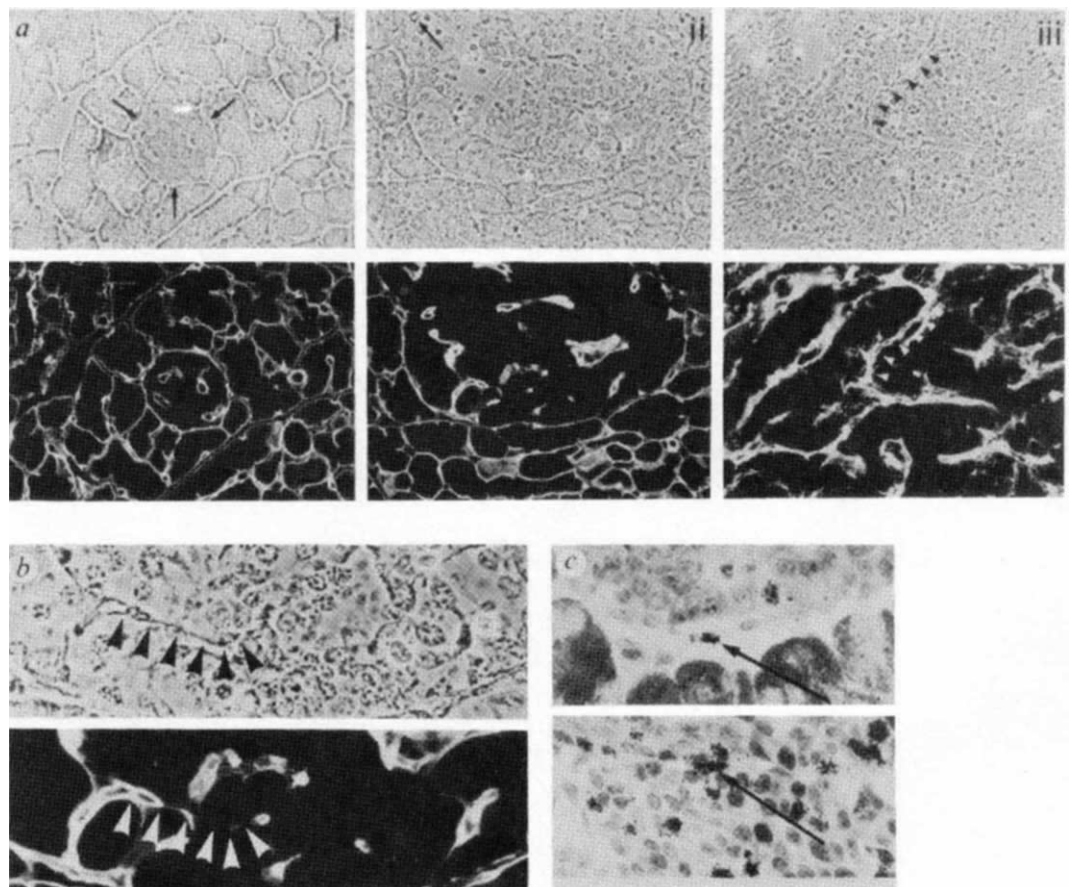
of neovascularization *in vivo*¹⁵ and *in vitro*¹⁶. Namely, the endothelial cells aligned in a radial pattern (Fig. 3d), then migrated towards the islet (Fig. 3e) and formed endothelial cell sprouts and occasional capillary tubes (Fig. 3f).

The assay also revealed that induction of angiogenesis is a local event which is selective or specific for the activated islet(s). Often, when multiple islets were cultured in the same well, one islet elicited capillary ingrowth, whereas its neighbours did not (Fig. 3c). There was no correlation between the size of a hyperplastic islet and its ability to induce capillary ingrowth. In some cases, an islet was angiogenic immediately upon introduction into the gel, whereas in other cases angiogenic activity arose only after a period of incubation *in vitro*, ranging from 3–12 days. These results in conjunction with our *in vivo* studies confirm that onset of angiogenic activity occurs in hyperplastic islets before overt tumour formation.

The ability to isolate hundreds of individual islets at defined times during progression to neoplasia has allowed us to determine the angiogenic capacity of each population in a statistical fashion, and to relate it to the frequency with which focal hyperplasias progress to neoplasia.

In an analysis of 1,900 islets, the percentage of transgenic islets that elicited angiogenesis *in vitro* was 0% at 4–5 weeks; $0.57 \pm 0.35\%$ at 6–7 weeks; $2.8 \pm 0.6\%$ at 8–10 weeks; and $3.6 \pm 0.39\%$ at 11–12 weeks (Table 1). As shown in Fig. 4, there was

FIG. 1 Characterization of neovascularization *in vivo*. Phase contrast (upper panels) and corresponding immunofluorescence views (lower panels) of paraffin sections through a normal islet (a(i)), a hyperplastic transgenic islet (a(ii), b) and a transgenic islet cell tumour (a(iii)). a, (i) The normal islet of Langerhans (indicated by arrows) appears as a well-demarcated circular structure that is surrounded by clusters of exocrine acini. Laminin appears in a continuous pattern within all basement membranes. Islet capillaries appear as brightly stained tubular structures. a, (ii) This transgenic hyperplastic islet appears enlarged but continues to retain well-defined boundaries. The small arrow indicates a mitotic figure within the islet parenchyma. Laminin appears in a linear basement membrane pattern surrounding certain capillaries in this hyperplastic islet, although other more pleomorphic capillaries exhibit wispy and fibrillar laminin distributions. b, A higher magnification view of the same hyperplastic islet shows a capillary sprout branching from a pre-existing islet capillary. Large arrowheads point to a single capillary endothelial cell that stretches from the end of a luminal cul-de-sac at the left and extends a long cell process to the right. The endothelial cell nucleus appears on the left between the first and second arrowheads at the blunt end of the capillary lumen. Laminin appears in a linear pattern within the capillary basement membrane and in a fine, fibrillar distribution in association with the extended cell process. Note that the endothelial cell sprout and capillary containing a lumen both share a common basement membrane. a, (iii) Normal islet architecture has become radically



disorganized and an extensive vascular network is evident within this islet cell tumour. The small arrowheads point to one tumour capillary. Laminin appears in both linear and diffuse, fibrillar patterns along tumour capillaries and connective tissue septae. Our method for immunostaining has been published previously¹¹. c, ^3H -thymidine autoradiographs showing induction of capillary endothelial cell DNA synthesis *in vivo*. Large arrows point to endothelial cell nuclei that exhibit autoradiographic grains within capillaries in a hyperplastic islet (upper), and in the parenchyma of an islet cell tumour (lower). (Magnifications: a, $\times 140$; b, $\times 350$; c, $\times 450$).

a striking correlation between the increasing incidence of transgenic islets that became angiogenic and the rising frequency of tumour formation. By contrast, the frequency with which hyperplastic islets developed was at all times much higher than the incidence of tumours. At 12 weeks, 75% of the islets were hyperplastic, whereas tumour incidence was only 1.5–2% (Fig. 4). Thus, it is the acquisition of angiogenic capacity by hyperplastic islets that scales with tumour incidence and not islet

hyperplasia *per se*. These data show that induction of angiogenic activity and consequent neovascularization both precede tumour formation and correlate with the transition from hyperplasia to neoplasia.

This experimental model probably has an important counterpart in human cancer. Most cancers originate from epithelial cells, for example, carcinoma of the bladder, breast, cervix, colon and skin. A discernible pre-neoplastic stage lasting several

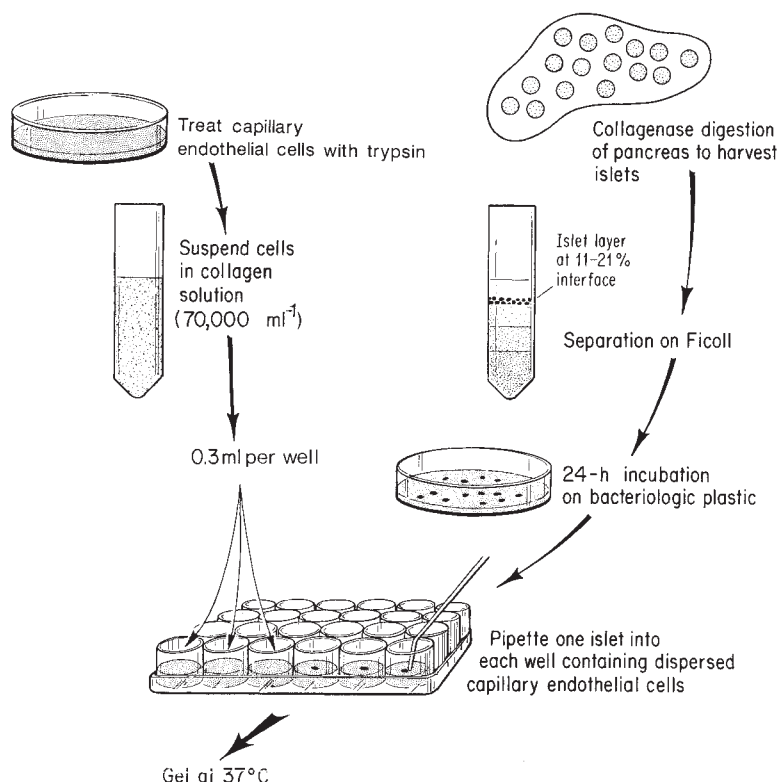


FIG. 2 *In vitro* gel assay for detection of angiogenic activity. Bovine capillary endothelial cells were cloned from adrenal cortex and passaged in gelatinized dishes as previously described²⁵. For *in vitro* detection of angiogenic activity, endothelial cells were treated with trypsin and resuspended in a chilled collagen gel solution composed of Vitrogen (Collagen Corporation) mixed 1:1 with RPMI medium +10% fetal bovine serum. Aliquots of the collagen-cell mixture were pipetted into 24-well tissue culture plates (Costar) and single pancreatic islets were added to each well before the solution was allowed to gel at 37 °C. Islets of Langerhans were harvested from mouse pancreases by intraductal injection of collagenase (Worthington IV CLS) as previously described¹⁴. Collagenase-digested pancreases were mechanically dissociated, passed through a stainless steel sieve (560 μ m mesh), and islets were separated from other cells and debris using a Ficoll gradient (layers of 25%, 23%, 21% and 11% Ficoll). Islets were collected from the 21%/11% Ficoll interface and stored for 1–2 days at 37 °C in a bacteriological culture dish containing RPMI medium +10% fetal bovine serum before use. Culture plates were incubated under 10% CO₂ and observed for endothelial cell reorientation, growth, migration, and differentiation (previous studies have shown that endothelial cells can form organized capillary tubes when grown on collagenous substrata^{25–27}).

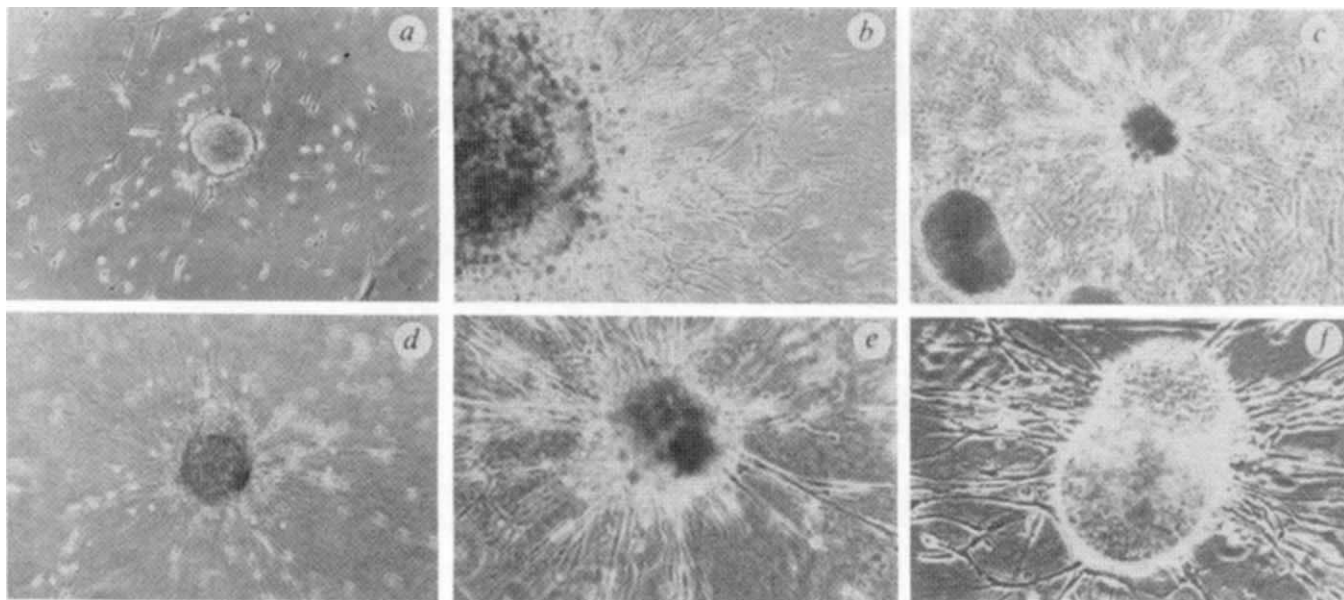


FIG. 3 *In vitro* assay of angiogenic activity during different stages in β -cell tumourigenesis. *a*, Normal islet from 14-week-old mouse exhibits a smooth border and is surrounded by randomly distributed capillary endothelial cells. *b*, Tumour from 12-week-old transgenic mouse induces extensive capillary endothelial cell ingrowth. *c*, 13-week-old hyperplastic islets. One islet in the centre is angiogenic, whereas the large islet at the bottom left is not. *d*, The initial response of endothelial cells to an angiogenic hyperplastic islet

is realignment in a radial pattern. *e*, Alignment is followed by locomotion of endothelial cells and migration in tandem. The smooth border of the islet is destroyed when the migrating endothelial cells contact its surface (higher magnification of *c*). *f*, Migration of endothelial cells toward the angiogenic hyperplastic islet is followed by development of organized capillary networks. Magnification: *a–d*, $\times 47$; *e, f*, $\times 93$.

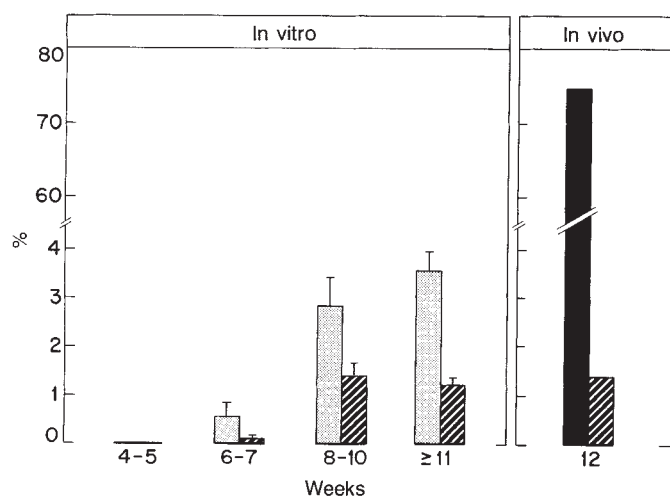


FIG. 4 Correlation of angiogenic activity with tumour incidence. The frequencies of angiogenic islets (stippled bars) and angiogenic tumours isolated *in vitro* (slashed bars, left panel) are compared with the incidence of hyperplastic islets (closed bar) and tumours *in vivo* (slashed bars, right panel). Tumours isolated *in vitro* are presented as a percentage of the approximate total number of islets present in each pancreas (400). The percentage of tumours present *in vivo* at 12 weeks is based upon routine autopsy of transgenic mice in this lineage. Islets are defined as hyperplastic *in vivo* if more than 20% of the β -cells incorporate ^3H -thymidine; the frequency of hyperplastic islets at 12 weeks is expressed as a percentage of total islets (from Teitelman *et al.*¹⁰).

years often precedes these malignancies¹⁷⁻²⁰. A distinguishing feature of most pre-neoplastic lesions is their lack of obvious neovascularization, as compared with the resulting neoplasias which are typically highly angiogenic. The 'switch' from the pre-vascular state to the vascularized stage may be followed by an increase in growth rate and metastasis²¹. Analysis of this switch has been limited by the unavailability of reproducible samplings of pre-neoplastic lesions at defined times during their development. This realization motivated previous investigators to examine angiogenesis during *de novo* tumour formation in animal models²²⁻²⁴. Unfortunately, the irregularity of tumorigenesis has limited the use of this approach, particularly with regard to isolating and analysing the pre-neoplastic stages.

By contrast, pre-neoplastic islets appear in a predictable manner in transgenic mice and are accessible to study. The development of an *in vitro* bioassay detects precisely when these islets switch to the angiogenic state. In conjunction with *in vivo* analysis, this bioassay provides an unprecedented and powerful new method for future studies into the molecular genetic events that govern the induction of angiogenesis and tumour progression.

The consensus of these and other studies on tumorigenesis in transgenic mice is consistent with a model in which oncogenes abrogate growth control and induce cell proliferation and consequent hyperplasia. This abnormal cell proliferation then sets the stage for additional genetic or epigenetic secondary events. Induction of angiogenesis seems to be one of these events that is important for the conversion of normal epithelium into a cancer. □

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ACKNOWLEDGEMENTS. This work was supported by grants to J.F. and to D.H. from the National Cancer Institute, and by grants from Takeda Chemical Industries, Ltd. (J.F.) and the Monsanto Company (D.H.). We thank Joan Alexander for mouse genetic analyses, Catherine Butterfield for cloning capillary endothelial cells and J. Madri of the Yale Medical School for providing laminin anti-sera. We thank Mary Brozna and Pauline Breen for typing, Steven Moscovitz for artwork and Lori DeSantos at Children's Hospital for photography.

Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1

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THE leukocyte adhesion molecule LFA-1 mediates a wide range of lymphocyte, monocyte, natural killer cell, and granulocyte interactions with other cells in immunity and inflammation^{1,2}. LFA-1 (CD11a/CD18) is a receptor for intercellular adhesion molecule 1 (ICAM-1, CD54), a surface molecule which is constitutively expressed on some tissues and induced on others in inflammation³⁻⁵. Induction of ICAM-1 on epithelial cells, endothelial cells and fibroblasts mediates LFA-1-dependent adhesion of lymphocytes^{4,6,7}. Several lines of evidence have suggested the existence of a second LFA-1 ligand: homotypic adhesion of one cell line was inhibited by a monoclonal antibody to LFA-1, but not by one to ICAM-1⁸; there exists an LFA-1-dependent, ICAM-1-independent pathway of adhesion to endothelial cells⁹; and also, there are some types of target cells in which LFA-1-dependent T-lymphocyte adhesion and lysis are independent of ICAM-1⁹. We have cloned this second ligand, designated ICAM-2, using a novel method for identifying ligands of adhesion molecules. ICAM-2 is an integral membrane protein with two immunoglobulin-like domains, whereas ICAM-1 has five^{10,11}. Remarkably, ICAM-2 is much more closely related to the two most N-terminal domains of ICAM-1 (34% identity) than either ICAM-1 or ICAM-2 is to other members of the immunoglobulin superfamily, demonstrating the existence of a subfamily of immunoglobulin-like ligands that bind the same integrin receptor.

We have developed a procedure for cloning functional adhesion molecules. LFA-1 purified in the presence of Mg^{2+} , which we have found to be functionally active in binding to both ICAM-1⁺ and ICAM-1⁻, putative second ligand⁺ cells (M.L.D. and T.A.S., manuscript in preparation), was coated on plastic. We used a modified version of the procedure of Aruffo and Seed for selecting complementary DNAs by expression in COS cells¹². To test the feasibility of this procedure, COS cells were transfected with the previously cloned ICAM-1 cDNA (Fig. 1a). ICAM-1 was expressed on 25% of the transfected COS cells. After panning, non-adherent cells were depleted of

Received 3 January; accepted 20 March 1989.

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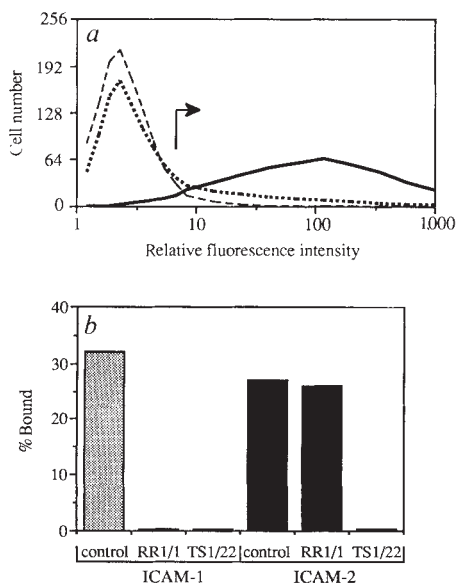


FIG. 1 Binding of transfected COS cells expressing ICAM-1 and ICAM-2 to LFA-1-coated plastic. *a*, COS cells transfected with ICAM-1 cDNA were panned on LFA-1-coated plates and the expression of ICAM-1 was analysed by indirect immunofluorescence flow cytometry with RR1/1 as the primary mAb. Unpanned cells, dotted line; non-adherent cells, dashed line; adherent cells, solid line. *b*, ^{51}Cr -labelled transfected COS cells expressing ICAM-1 or ICAM-2 were bound to LFA-1-coated plastic in the presence of mAb.

METHODS. LFA-1 was purified from SKW-3 lysates²⁴ by immunoaffinity chromatography on TS2/4 LFA-1 mAb Sepharose and eluted at pH 11.5 in the presence of 2 mM MgCl_2 and 1% octylglucoside (M.L.D. and T.A.S., manuscript in preparation). LFA-1 (10 μg per 200 μl per 6-cm plate) was bound to bacteriological Petri dishes by diluting octylglucoside to 0.1% in PBS with 2 mM MgCl_2 and overnight incubation at 4 °C. Plates were blocked with 1% BSA and stored in PBS/2 mM MgCl_2 /0.2% BSA/0.025% azide/50 μg ml^{-1} gentamycin. Synthesis of a cDNA library from lipopolysaccharide-stimulated umbilical vein endothelial cells was as described previously¹⁰. After second-strand synthesis, the cDNA was ligated to *Bst*XI adaptors¹² and cDNAs longer than 600 bp were selected by low-melting point agarose gel electrophoresis. The cDNA was then ligated to CDM8 (ref. 25), introduced into *E. coli* host MC1061/P3 and plated to obtain 5×10^5 colonies. The colonies were suspended in LB medium, pooled and plasmid prepared by standard alkali-lysis method²⁶. Ten 10-cm plates of COS cells at 50% confluency were transfected with 10 μg per plate of the plasmid cDNA library using DEAE-dextran²⁷. The putative second LFA-1 ligand is trypsin-resistant on endothelial and SKW-3 cells (M.L.D., unpublished results). COS cells three days after transfection were suspended by treatment with 0.025% trypsin/1 mM EDTA/HBSS (Gibco) and panned¹² on LFA-1 coated plates as described below for ^{51}Cr -labelled COS cells. Adherent cells were released by addition of 10 mM EDTA. Plasmid was recovered from the adherent population of COS cells in Hirt supernatants²⁸. The *E. coli* strain MC1061/P3 was then transformed with the plasmid, colonies on plates were suspended in LB medium, pooled and plasmid prepared by the alkali-lysis method. Selection of LFA-1-adherent transfected COS cells and plasmid recovery was repeated twice. Pooled colonies obtained after the third cycle were grown to saturation in 100 ml LB medium with 18 μg ml^{-1} tetracycline and 20 μg ml^{-1} ampicillin. Plasmid was prepared and fractionated by 1% low-melting point agarose gel electrophoresis and MC1061/p3 was transformed separately with plasmid from nine different size fractions. Individual plasmids from the fraction with greatest activity in promoting COS cell adhesion to LFA-1 were examined for uniqueness by restriction enzyme digestion and tested in the COS cell adherence assay. This yielded one plasmid with an ICAM-2 cDNA insert of 1.1 kb, pCDIC2.27. For adhesion assays, the ICAM-2 plasmid pCDIC2.27 or an ICAM-1 construct containing the 1.8-kb *Sall*-*KpnI* fragment¹⁰ in CDM8 (2 μg per 10-cm plate) was introduced into COS cells using DEAE-Dextran. COS cells were suspended with 0.025% trypsin/1 mM EDTA/HBSS 3 days after transfection and labelled with ^{51}Cr . Approximately 2×10^5 ^{51}Cr -labelled COS cells in 2 ml PBS/5% FCS/2 mM MgCl_2 /0.025% azide (buffer) with 5 μg ml^{-1} of the mAb indicated were incubated in LFA-1-coated 6-cm plates at 25 °C for 1 hour. Non-adherent cells were removed by gentle rocking and three washes with buffer. Adherent cells were eluted by the addition of EDTA to 10 mM and γ -counted.

ICAM-1⁺ cells, whereas adherent cells released from LFA-1-coated plastic by EDTA were almost completely ICAM-1⁺. Adherence of ICAM-1⁺ cells to LFA-1-coated plastic was inhibited with the ICAM-1 monoclonal antibody (mAb) RR1/1. LFA-1 coated on Petri dishes was stable for more than five cycles of COS cell adherence and elution with EDTA.

To clone the second LFA-1 ligand, a cDNA library in the plasmid vector CDM8 was prepared from endothelial cells, which use both the ICAM-1-dependent and ICAM-1-independent components of LFA-1-dependent adhesion⁶. Transfected COS cells were incubated in LFA-1-coated Petri dishes with ICAM-1 mAb present to prevent isolation of ICAM-1 cDNA. Adherent cells were eluted with EDTA and plasmids were isolated and amplified in *Escherichia coli*. After three cycles of transfection, adherence and plasmid isolation and one size fractionation, 30 plasmids were analysed by restriction endonuclease digestion. Of three whose inserts were more than 1 kilobase (kb), one plasmid introduced into COS cells by transfection caused adherence to LFA-1.

This plasmid conferred adherence to LFA-1 in a high percentage of the transfected cells, similar to the percentage seen with ICAM-1 transfection (Fig. 1*b*). Adherence was blocked by LFA-1 mAb, but not by ICAM-1 mAb, in contrast to ICAM-1 transfectants (Fig. 1*b*). Furthermore, cells transfected with this plasmid did not react with a panel of four ICAM-1 mAbs. Thus, all functional criteria for a cDNA encoding a second LFA-1 ligand were fulfilled, and we designated this ligand ICAM-2.

The ICAM-2 cDNA sequence of 1,052 base pairs (Fig. 2) contains a 62-bp 5' untranslated region and a 165-bp 3' untranslated region. An AATACA polyadenylation signal at position 1,022, which, in contrast to AATAAA, occurs in approximately 2% of vertebrate messenger RNAs¹³, is followed at 1,041 bp by a poly(A) tail. The longest open reading frame begins with the first ATG at position 63 and ends with a TAG termination codon at position 885. Hydrophobicity analysis¹⁴ and usage of amino acids around cleavage sites¹⁵ predict a 21-residue signal peptide (Fig. 2). The predicted mature sequence contains a putative extracellular domain between amino acids 1 and 202, followed by a 26-residue hydrophobic putative transmembrane domain and a 26-residue cytoplasmic domain. Four turns of the putatively α -helical transmembrane segment are amphipathic, with threonine and serine residues falling on one side, suggesting the possibility of self-association or association with other membrane proteins in the plane of the membrane. The cytoplasmic domain is unusually basic. The predicted relative molecular mass (M_r) of the mature polypeptide is 28,393 which, if the six predicted *N*-linked glycosylation sites are used, would result in a ICAM-2 glycoprotein of M_r about 46,000.

The 1.1-kb ICAM-2 cDNA hybridizes to a 1.4-kb poly(A)⁺ mRNA and weakly to a 3-kb mRNA (Fig. 3*a*), both of which are distinct from the 3.3-kb and 2.4-kb ICAM-1 mRNA (Fig. 3*b*). We examined mRNA in cells that have been characterized functionally for ICAM-1-dependent and second-ligand-dependent binding to LFA-1. ICAM-1 mRNA is strongly induced in endothelial cells by lipopolysaccharide (Fig. 3*b*, lanes 2 and 3). In contrast, the basal expression of ICAM-2 mRNA is high in endothelial cells and is not induced further by lipopolysaccharide (Fig. 3*a*, lanes 2 and 3). This correlates with the strong basal and non-inducible expression of the LFA-1-dependent, ICAM-1-independent, pathway in endothelial cells and the inducibility of the ICAM-1-dependent pathway⁶. ICAM-2 mRNA is present in a wide variety of cell types including Ramos and BBN B lymphoblastoid, U937 monocytic and SKW3 T lymphoblastoid cell lines (Fig. 3*a*: lanes 1, 4, 6 and 8, moderate or long autoradiograph exposure). Of these, SKW3, U937 and BBN have been shown to adhere to LFA-1⁺ cells in an LFA-1-dependent, ICAM-1-independent way^{8,9}, and to LFA-1-coated plastic (M.L.D., unpublished results). The HeLa epithelial cell line, which has only the ICAM-1-dependent component of LFA-1-dependent adhesion⁹, shows no ICAM-2 mRNA (Fig.

[illegible]

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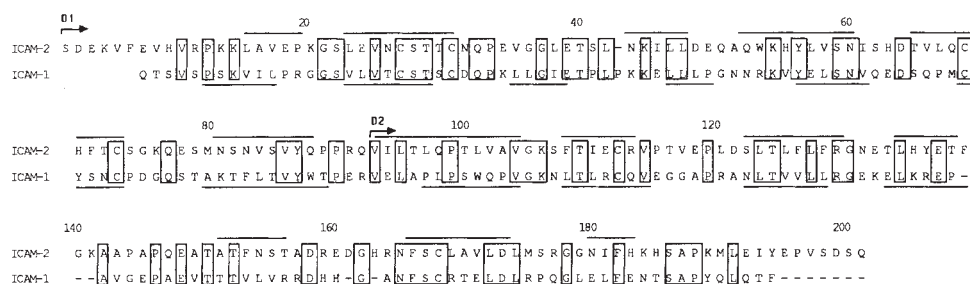


FIG. 4 ICAM-2 homology to ICAM-1. The entire 202-residue extracellular sequence of ICAM-2 was aligned with ICAM-1 residues 1–185 using the ALIGN program³⁰ and by inspection. ICAM-2 residues are numbered; identities are boxed. D1 and D2 indicate the boundary of immunoglobulin-like domains of ICAM-2 and ICAM-1. β -strand predictions³¹ of ICAM-2 are overlined and those of ICAM-1 are underlined.

is both structural and functional. Although there is little precedence among cell adhesion receptors, several integrin receptors for extracellular matrix components have been shown to recognize multiple ligands^{19,20}. Neither ICAM-1 or ICAM-2 contains an Arg-Gly-Asp (RGD) sequence, and thus the mode of recognition by LFA-1 may differ from integrins that bind extracellular matrix components^{19,20}. It will be interesting to determine whether the cellular ligands recognized by Mac-1 and p150,95, leukocyte integrins closely related to LFA-1², belong to the same immunoglobulin subfamily. ICAM-1 has recently been shown to be a receptor for the major group of rhinoviruses which cause 50% of common colds^{21,22}, whether ICAM-2 also functions as a receptor for rhinoviruses or other picornaviruses remains to be determined.

The identification of a family of LFA-1 ligands emphasizes the importance of this recognition pathway and may be a mechanism for imparting fine specificity and functional diversity. Several differences between ICAM-1 and ICAM-2 are of potential importance. ICAM-1 is inducible on most cells, whereas ICAM2 expression is not affected by cytokines on the cells thus far tested. The three additional domains on ICAM-1 are expected to project its LFA-1 binding site further from the cell surface than that of ICAM-2, suggesting that closer cell-cell contact would be required for the LFA-1/ICAM-2 than the LFA-1/ICAM-1 interaction. ICAM-2-transfected COS cells are more readily detached from LFA-1-coated plastic, compared with ICAM-1 transfected COS cells, as the washing shear force is increased. This may be due to the smaller size of ICAM-2 which may make it less accessible to LFA-1 on the artificial substrate, or to differences in sequence which impart differences in affinity. LFA-1 and ICAM-1 are receptors for each other²³, and although we have arbitrarily referred to LFA-1 as a receptor and to the ICAMs as ligands, both are cell surface molecules and may function as signalling receptors which inform cells of their environments. Thus, the distinct cytoplasmic domains of ICAM-1 and ICAM-2 may impart different signals or may cause differing localization on the cell surface; likewise, signalling or interaction with the cytoskeleton by LFA-1 may differ depending on whether ICAM-1 or ICAM-2 is bound. □

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ACKNOWLEDGEMENTS. This work was supported by the NIH and Boehringer-Ingelheim. We thank B. Seed for COS cells and the CDM8 vector.

Mast cell lines produce lymphokines in response to cross-linkage of Fc ϵ RI or to calcium ionophores

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THE cross-linkage of high affinity Fc ϵ receptors (Fc ϵ RI) on mast cells and basophils is central to the induction of allergic inflammatory responses. As a result of such cross-linkage, mast cells secrete a variety of preformed biologically active substances, such as histamine, and newly synthesized arachidonic acid metabolites¹. Here we show that cross-linkage of Fc ϵ RI on a series of non-transformed murine mast cell lines, or treatment of these cells with calcium ionophores, stimulates increased messenger RNA levels and secretion of a group of lymphokines classically produced by a subset of murine T cell lines (T_H2 cells)². These factors include interleukin-3 (a mast cell growth factor), interleukin-4 (an IgE 'switch factor'), interleukin-5 (an eosinophil differentiation factor) and interleukin-6 (a factor controlling immunoglobulin secretion). The production of these polypeptide factors by mast cells may have great importance in the induction of allergic and anti-parasite inflammatory responses.

The murine mast cell lines CFTL 12, CFTL 15 and CFTL 17 were obtained by culturing fetal liver from NFS/N mice in interleukin-3 (IL-3) (ref. 3). These lines are dependent upon IL-3 for their growth and are propagated as homogeneous cell populations. They possess properties similar to mast cells from the intestinal mucosa, but different from connective tissue-type (for example, peritoneal) mast cells⁴. For most of these studies we used cells of the CFTL 12 and CFTL 15 lines, which have

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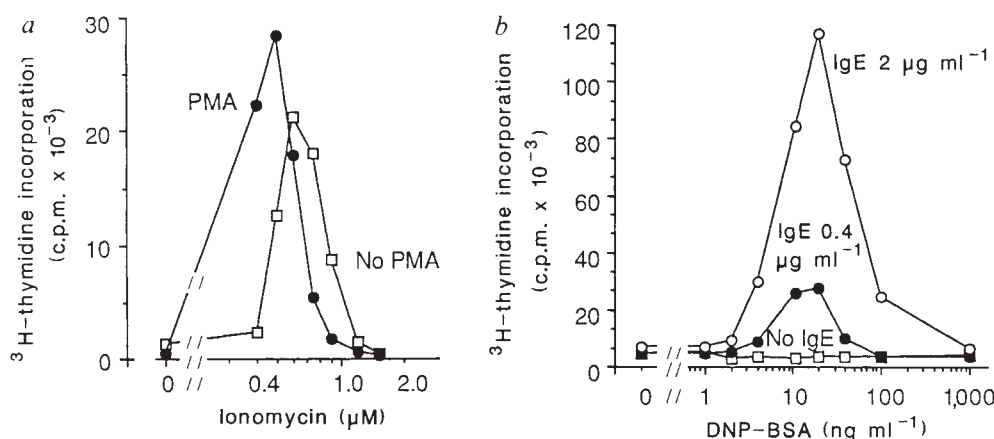


FIG. 1 *a*, Ionomycin-induced DNA synthesis by CFTL 12. Cells were incubated (50,000 per 0.2 ml per well) in RPMI 1640 with penicillin/streptomycin, L-glutamine, 2-mercaptoethanol, non-essential amino acids, and 10% fetal calf serum, with ionomycin alone (open squares), or with ionomycin and 1 ng ml⁻¹ phorbol myristate acetate (closed circles) for 60 h, in 96-well flat-bottomed microtitre plates (Costar), with a terminal 12 h pulse with 1 μCi ³H-thymidine (6.7 Ci mmol⁻¹, ICN). *b*, Antigen-induced DNA synthesis by CFTL 12. Cells were sensitized by incubation for 2 h with 0 (open squares), 0.4 (closed circles) or 2 (open circles) μg ml⁻¹ IgE anti-DNP, then washed and incubated with DNP₂₃BSA for 72 h with a terminal 6 h pulse with [³H]thymidine.

been in culture for more than a year. We also performed several experiments with clones derived from CFTL 12, with CFTL 17, a newly isolated NFS/N line, in culture for less than 4 months, and with the PT-18 cell line, cloned from C3H.SW spleen by Dr Dov Pluznik⁵. One of these lines, CFTL 12, grows in the presence of either IL-3 or IL-4.

We observed that the treatment of CFTL 12 cells with the calcium ionophores ionomycin or A23187 led to a striking uptake of [³H]thymidine by these cells in the absence of any exogenous source of IL-3 or IL-4. The response to ionomycin was dose-dependent and was inhibited at high concentrations of ionomycin (Fig. 1*a*). Although phorbol myristate acetate by itself did not induce DNA synthesis by CFTL 12 cells, it altered the ionomycin dose-response curve so that lower concentrations were required both for stimulation and inhibition of DNA synthesis. A23187 elicited a similar response, but the concentration range over which its growth stimulatory activity was mediated was narrower than that of ionomycin. The uptake of [³H]thymidine by CFTL 12 cells in response to ionomycin was inhibited by cyclosporine A (ref. 6) at a concentration of 50 ng ml⁻¹ (data not shown), suggesting that ionomycin elicits its stimulation of CFTL 12 growth by causing lymphokine synthesis. Ionomycin also stimulated DNA synthesis by CFTL 15, CFTL 17 and PT-18 cells (data not shown).

Cross-linkage of FcεRI has been shown to lead to elevation of cytosolic calcium¹. We therefore investigated whether antigen-mediated crosslinking of IgE receptors could also induce DNA synthesis. CFTL 12 mast cells, which express 100,000 FcεRI with an affinity similar to that of rat basophil leukaemia cells, were sensitized with a monoclonal IgE anti-DNP antibody (Hi-DNP-ε-26; ref. 7), washed and cultured with dinitrophenylated-bovine serum albumin (DNP-BSA) containing an average of 23 DNP groups per BSA molecule. DNP-BSA induced no uptake of [³H]thymidine in unsensitized CFTL 12 cells, but CFTL 12 cells sensitized with 0.4 or 2.0 μg ml⁻¹ IgE antibody exhibited striking uptake of [³H]thymidine in response to DNP-BSA in a narrow concentration range (5–100 ng ml⁻¹, Fig. 1*b*) and to monoclonal rat anti-mouse IgE (B1E3). Antigen and monoclonal anti-IgE also stimulated proliferation of IgE-sensitized CFTL 15, CFTL 17 and PT-18 cells (data not shown). DNP-lysine (10⁻⁶ M) completely inhibited DNP-BSA-induced [³H]thymidine incorporation by sensitized CFTL 12 cells but had no effect (up to 10⁻⁴ M) on responses to anti-IgE. DNP-lysine, by itself, had no stimulatory effect on sensitized CFTL 12 cells. These results suggest that cross-linkage of FcεRI by antigen induces autocrine production of growth factors for mast cells. To determine whether the proliferative response of CFTL 12 was due to production of IL-3 and IL-4, neutralizing antibodies to these cytokines were tested and were found to be inhibitory. Anti-IL-3 (ref. 8) and anti-IL-4 (ref. 9) monoclonal antibodies used together fully inhibited proliferation of ionomy-

cin-stimulated cells, and anti-IL-3 alone fully inhibited antigen-induced proliferation (data not shown). Moreover, all of the tested mast cell lines secreted IL-3 when stimulated by ionomycin or by cross-linkage of FcεRI (Table 1). In response to 1 μM ionomycin, CFTL 12 cells (2 × 10⁶ ml⁻¹) secreted up to 20,000 U ml⁻¹ of IL-3, an amount comparable to that produced by cultures of WEHI-3 cells. This concentration of ionomycin was substantially higher than that required for maximal [³H]thymidine uptake (Fig. 1), suggesting that elevated levels of ionomycin inhibit CFTL 12 growth either as a result of high cytosolic [Ca²⁺] or through the production of an inhibitory substance, rather than by failure of production of growth factors. The amount of IL-3 produced by CFTL 12 in response to FcεRI cross-linkage was ~50-fold less than that observed in response to ionomycin but was still quite substantial.

Mast cells sensitized with IgE anti-DNP exhibited antigen (DNP₂₃BSA) dose-dependent secretion of IL-3 marked by peak release at 30 to 100 ng ml⁻¹ and complete inhibition at

TABLE 1 Production of lymphokines by IgE-sensitized mast cell lines

Cell line	Stimulus	Lymphokine secreted (U ml ⁻¹)				
		IL-2	IL-3	IL-4	IL-6	INF-γ
CFTL 12	0	<1	<10	<15	<10	<1
	Antigen	<1	250	<15	790	<1
	Ionomycin	<5	19,000	100	6,600	<1
CFTL 15	0	<1	<10	<15	<10	<1
	Antigen	<1	1,500	280	14,000	<1
	Ionomycin	<5	1,800	32	1,300	<1

Mast cell lines were sensitized by incubating for 3 h with 3 μg ml⁻¹ IgE anti-DNP, then washed and incubated (2.5 × 10⁶ cells per ml) for 6 h with buffer only ('0'), 100 ng ml⁻¹ DNP₂₃BSA ('antigen') or 1.2 μM ionomycin. Cell-free supernatants, obtained by centrifugation, were maintained at 4 °C until assayed for lymphokines as follows. IL-2: ³H-thymidine incorporation over 36 h by 5,000 CTLL cells per well, in the presence of 2 μg ml⁻¹ anti-IL-4 antibody (11B11, ref. 9); sensitivity 0.5 U ml⁻¹. IL-3: ³H-thymidine incorporation over 24 h by 10,000 FD.C/1 cells, in the presence of anti-IL-4 antibody, and inhibition by monoclonal rat anti-IL-3 antibody (19B3.1, ref. 8); sensitivity 1 U ml⁻¹ (based on Genzyme purified IL-3). IL-4: ³H-thymidine incorporation over 60 h by 5,000 CT.4S cells per well²⁹, and its blockade by 2 μg ml⁻¹ 11B11; sensitivity 3 U ml⁻¹. IL-6: measured as hybridoma growth factor units, by ³H-thymidine incorporation over 88 h by 2,000 B9 cells³⁰, and inhibition by 10 μg ml⁻¹ rabbit anti-IL-6 polyclonal antibody; sensitivity 1 U ml⁻¹. INF-γ: inhibition of growth of vesicular stomatitis virus (VSV-1) on L929/261 cells; sensitivity 1 U ml⁻¹ (that is, 1 pg ml⁻¹). The cell lines CFTL 17 and PT-18 were tested in similar experiments for IL-3 and IL-4 production. Unstimulated CFTL 17 and PT-18 secreted undetectable levels of IL-3 and IL-4. Antigen-stimulated CFTL 17 secreted 720 U ml⁻¹ IL-3 and 38 U ml⁻¹ IL-4; ionomycin-stimulated CFTL 17 secreted 2,700 U ml⁻¹ IL-3 and 130 U ml⁻¹ IL-4. Antigen-stimulated PT-18 secreted 20 U ml⁻¹ IL-3, and ionomycin-stimulated PT-18 secreted 1,500 U ml⁻¹ IL-3; PT-18 did not secrete detectable levels of IL-4.

300 ng ml⁻¹. Histamine release also was optimal between 30 and 100 ng ml⁻¹ (Fig. 2). In the experiment illustrated, 300 ng ml⁻¹ was not inhibitory, but in other experiments (data not shown) 500 to 1,000 ng ml⁻¹ inhibited histamine release. DNP-lysine (10⁻⁵ M) completely inhibited IL-3 production by sensitized CFTL 12 in response to DNP-BSA but had no effect on IL-3 production in response to anti-IgE. Histamine secretion occurs from preformed stores in granules¹ and occurs within minutes of cross-linkage of FcεRI. By contrast, IL-3 secretion was first detected at one hour and reached maximal levels at two to six hours after DNP-BSA addition to sensitized CFTL 12 cells.

Table 1 shows the results of investigating the production of other lymphokines. Ionomycin stimulated CFTL 12 and CFTL 15 to secrete high levels of IL-4. Antigen-stimulated IgE-sensitized CFTL 15 cells produced up to 400 U ml⁻¹ of IL-4, whereas sensitized CFTL 17 cells produced lesser amounts, and sensitized CFTL 12 and PT-18 cells failed to secrete detectable amounts of IL-4 in response to antigen. IL-6 production was stimulated in CFTL 12 and CFTL 15 by both antigen and ionomycin. By contrast, CFTL 12 and CFTL 15 did not secrete either IL-2 or IFNγ in response to either antigen or ionomycin. Because of the high concentrations of other lymphokines in mast cell supernatants, we have not yet been able to demonstrate conclusively that the supernatants contain IL-5.

Mast cells store a variety of mediators, which are secreted upon appropriate stimulation such as cross-linkage of FcεRI. To determine whether lymphokines are newly produced or are from preformed stores and to verify the biological identification of these lymphokines, we examined the expression of lymphokine mRNA in IgE-sensitized CFTL 12 cells stimulated with antigen or ionomycin. Poly(A)⁺ RNA was prepared from CFTL

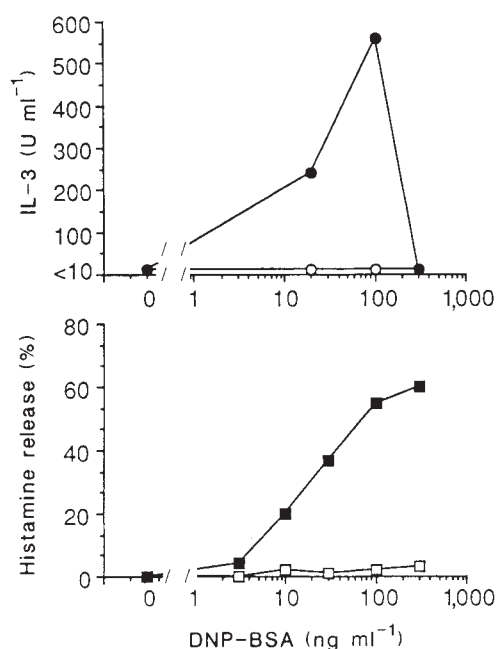


FIG. 2 Antigen-stimulated histamine and IL-3 secretion from CFTL 12 cells. Cells were sensitized by incubation for 2 h with 0 (open symbols) or 2 μg ml⁻¹ (closed symbols) IgE anti-DNP, then incubated with DNP₂₃BSA. The results for IL-3 and histamine shown here are from two sequential experiments. To measure IL-3, the mast cells were cultured (2 × 10⁶ per ml, 18 h) in RPMI 1640-FCS, and supernatants were collected. IL-3 was measured by the growth of FD.C/1 cells, as described in Table 1. To measure histamine, mast cells were cultured (10⁶ per ml, 45 min) at 37 °C in 0.5 ml, with activating agents in PIPES buffer (pH 7.4) containing 1 mM Ca²⁺, 1 mM Mg²⁺ and 0.1% gelatin. Total histamine content was obtained by lysing cells with 1.6% perchloric acid. Histamine was assayed by an automated spectrofluorometer³¹ and per cent release calculated after correcting for spontaneous histamine secretion (with buffer alone), which was 5% of the total.

TABLE 2 Lymphokine production by mouse T cells and mast cell lines

Lymphokine	T _{H1}	T _{H2}	Mast cell
IL-3	++	++	++
GM-CSF	++	++	ND
TNFα	++	++	0*
IL-4	0	++	++
IL-5	0	++	++
IL-6	0	++	++
IL-2	++	0	±
IFN-γ	++	0	±
LT	++	0	0

Refs 2, 11 and 12 document the T_{H1} versus T_{H2} source of these lymphokines. ND, not determined.

* Biological activity but no mRNA.

12 cells two hours after stimulation and tested by northern analysis. Resting cells did not express active, 0.9-kilobase (kb) mRNA for IL-3 (Fig. 3a), whereas antigen-stimulated cells expressed detectable levels and ionomycin-stimulated cells expressed very large amounts of IL-3 mRNA. The significance of the 2 kb mRNA seen in unstimulated mast cells and, less intensely, in antigen-stimulated cells, is being investigated. Resting mast cells expressed low levels of IL-4 mRNA and IL-6 mRNA and no detectable IL-5 mRNA (Fig. 3b-d). Cross-linkage of FcεRI on CFTL 12 cells caused a modest increase in IL-4 mRNA and substantial increases in IL-5 and IL-6 mRNA. Ionomycin-stimulated cells expressed large amounts of IL-4, IL-5 and IL-6 mRNA, so that even 10 μg of total, unselected RNA gave a strong signal (not shown). By contrast, neither resting nor antigen-stimulated mast cells expressed IL-2, interferon-γ (IFNγ), lymphotoxin (LT; TNFβ) or tumour necrosis factor (TNFα) mRNA (Fig. 3e-f). Ionomycin-stimulated cells expressed small but detectable amounts of the mRNA species for IL-2 and IFNγ (note the 63 h film exposure required to detect IFNγ), but no detectable TNFα or LT mRNA.

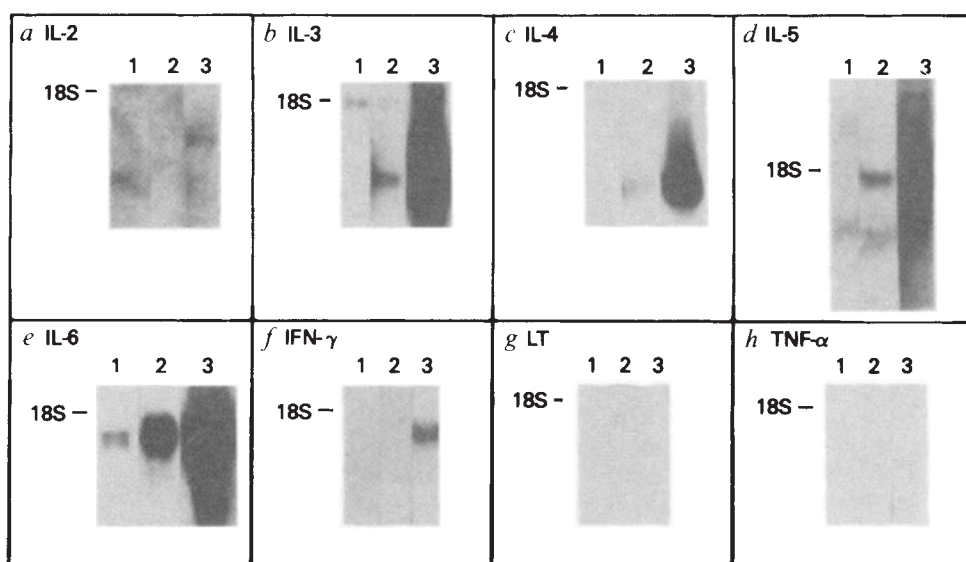
These results indicate that non-transformed long-term mast cell lines produce a set of lymphokines, generally considered to be principally derived from T cells, as a result either of cross-linking of FcεRI or exposure to calcium ionophores. It has been previously reported that a myeloid cell line (FD.C/1) produces IL-3 and IL-4 in response to immune complexes¹⁰ and recent work (M.P. and G. S. LeGros, unpublished observations) indicates that this stimulation depends upon cross-linkage of the FcγRII, suggesting that myeloid cells may share with mast cells the property of stimulation by cross-linkage of receptors specific for Fc regions of immunoglobulin.

It is particularly striking that the set of lymphokines produced by stimulated mast cells closely matches the set of these polypeptides produced by long-term murine T-cell lines of the T_{H2} type². T_{H2} cells produce IL-4 and IL-5 (ref. 11), as well as IL-6 (ref. 12) (which is also produced by a variety of other cell types¹³) but not IL-2, IFNγ or LT which are made by long-term T-cell lines of the T_{H1} type. Both T_{H1} and T_{H2} cells produce IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF), and have also been shown to secrete TNFα. Although we do not observe TNFα mRNA in CFTL 12, it has previously been reported and we have confirmed that mast cells can secrete a molecule with antigenic relatedness to TNF and with some biological properties similar to TNF and to the cytotoxic T cell-derived factor 'leukolexin'¹⁴⁻¹⁵.

The lymphokines produced by stimulated mast cells and by T_{H1} and T_{H2} cell lines are summarized in Table 2. Those made by both mast cell lines and T_{H2} cells appear well adapted to participate in allergic inflammatory responses.² IL-4 has been clearly shown to be critical for IgE production both *in vitro*¹⁶ and *in vivo*¹⁷; IL-5 is a potent eosinophil differentiation and activation factor¹⁸; and both IL-3 (ref. 19) and IL-4 (ref. 20)

FIG. 3 Northern analysis of CFTL 12-derived poly(A)⁺ RNA, probed with complementary DNA fragments for (a) IL-2, (b) IL-3, (c) IL-4, (d) IL-5, (e) IL-6 (f) interferon- γ (IFN- γ), (g) lymphotoxin (LT) (TNF β), (h) tumour necrosis factor (TNF α). Cells were sensitized by incubating with 2 μ g ml⁻¹ IgE anti-DNP for 2 h, then cultured for 2.5 h with (lane 1) buffer alone, (lane 2) 100 ng ml⁻¹ DNP₂₅BSA, or (lane 3) 1.2 μ M ionomycin before preparing RNA. Comparable results were obtained in two separate experiments: panels a-d are from experiment 1; panels e-h are from experiment 2. All blots were from electrophoresis of 5 μ g poly (A)⁺ RNA, except for those probed with IL-2 and IL-5, in which only 2 μ g samples were run. The duration of film exposure was as follows: IL-2, 24 h; IL-3, 6 h; IL-4, 6 h; IL-5, 24 h; IL-6, 12 h; IFN- γ , 63 h; LT, 96 h; TNF α , 96 h.

METHODS. The cells were washed, lysed in NP-40, centrifuged free of nuclei, and the cytoplasmic RNA was purified with phenol-chloroform-isoamyl alcohol³². Poly (A)⁺ RNA was selected by two passages over an oligo(dT) column (Collaborative Research), electrophoresed on a 1% agarose-formaldehyde gel, and transferred to nitrocellulose or (for IL-6) nylon (Nytran, Schleicher and Schuell). The blots were probed with fragments of lymphokine cDNA, labelled by the random hexamer method³³. 200 ng was incubated for 4 h at 23 ° with 50 μ Ci of ³²P-dCTP and passed twice over Sephadex G-50 columns (Boehringer) to remove free nucleotide, yielding approximately 5 $\times$ 10⁷ c.p.m. Following overnight hybridization (1–2 $\times$ 10⁷ c.p.m. ml⁻¹, 0.1 ml per cm² nitrocellulose or nylon), the blot was washed three times at room temperature and then for



30 min under stringent conditions (0.1 $\times$ SSC; 0.1% SDS for nitrocellulose, 0.5% SDS for nylon; 65 °C) and exposed to Kodak XAR film at –70 °C, with an intensifying screen. The probes used were as follows: IL-2, 600 base pair (bp) *Pst* I fragment of IL-2 cDNA (ref. 34); IL-3, 600 bp *Pst* I fragment of IL-3 cDNA (ref. 3); IL-4, 424 bp *Eco*RI-*Hind* III fragment which includes the 373 bp *Rsa* I fragment of IL-4 cDNA and 51 bp of pGEM-3 (ref. 3); IL-5, 650 bp *Eco*RI-*Hind* III fragment of IL-5 cDNA (ref. 35); IL-6, 950 bp *Xho* I fragment of IL-6 cDNA (ref. 36); IFN- γ , 643 bp *Pst* I fragment of γ -IFN cDNA (ref. 37); LT, 800 bp *Kpn* I-*Hinc* II fragment of LT cDNA (ref. 38); TNF α , the 1,400 bp *Pst* I-*Bam* HI fragment of TNF α cDNA (ref. 39).

can stimulate growth of mast cells, possibly of both the mucosal and connective tissue types²¹. Our findings are therefore consistent with the idea that normal mast cells play an important role in allergic and anti-parasite responses, perhaps by strongly amplifying the signals normally mediated by T cells. T cells may secrete lymphokines only in the context of intimate contacts between themselves and antigen-presenting cells²² and the action of these lymphokines may be largely restricted to the cells with which they interact. By contrast, mast cells sensitized with IgE could mediate systemic reactions by releasing these lymphokines in response to Fc ϵ RI cross-linkage into the extracellular fluid in a way that ensures access to a wide variety of target cells. We have evidence that normal mast cells produce lymphokines, in that purified peritoneal mast cells from CBA/J mice sensitized with IgE anti-DNP secrete IL-3 in response to anti-IgE (M.P., unpublished observations). Mast cell numbers are increased in rodent infections with *Nippostrongylus brasiliensis*²³ and in certain human inflammatory diseases²⁴, suggesting that such cells, and/or their precursors, may strikingly amplify the production of lymphokines in these disorders. Furthermore, allergic reactions are not characterized simply by mast cell degranulation. Experimentally induced allergic responses are also marked by a so-called late phase reaction, occurring hours after the initiation of the reaction, which is widely believed to mimic inflammatory responses in clinical allergy^{25–27}. The predominant cellular component of exudates in late phase reaction is the activated eosinophil²⁸. We speculate that lymphokines secreted by activated mast cells may account for late phase reaction and for much of the inflammatory reaction in allergic diseases. □

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ACKNOWLEDGEMENTS. We thank Henry Metzger and Stephen Dreskin for IgE receptor determinations, IgE anti-DNP, DNP-BSA, and for helpful advice; Nancy Ruddle for probing mRNA for lymphotoxin and TNF α ; Daniel H. Conrad for IgE anti-DNP and monoclonal anti-IgE; Teruko Ishizaka and Dov Pluznik, for PT-18 cells; Tasuku Honjo for IL-5 cDNA; Patrick Grey for interferon- γ cDNA; John Abrams, for anti-IL-3 antibody; and Anne Kagey-Sobotka and Lawrence M. Lichtenstein for histamine measurements and helpful advice. The work was supported, in part, by the SCOR programme of the National Heart, Lung and Blood Institute, NIH, and the Johns Hopkins University School of Medicine; much of it was performed while Dr Plaut was on sabbatical leave at the Laboratory of Immunology, NIAID, NIH. This is publication no. 752 of the O'Neill Research Laboratories.

Received 22 February; accepted 17 March 1989.

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Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules

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THE human immunodeficiency virus type 1 (HIV-1) exploits the cell surface CD4 molecule to initiate the infection^{1–4} which can lead, eventually, to acquired immunodeficiency syndrome (AIDS). The HIV-1 envelope protein, gp120, interacts specifically with CD4 and soluble CD4 molecules have been shown to inhibit HIV infectivity *in vitro*^{5–9}. Effective inhibition *in vivo* may, however, require more potent reagents. We describe here the generation of molecules which combine the specificity of CD4 and the effector functions of different immunoglobulin subclasses. Replacing the VH and CH1 domains of either mouse γ 2a or μ heavy chains with the first two N-terminal domains of CD4 results in molecules that are secreted in the absence of any immunoglobulin light chains. We find that the pentameric CD4–IgM chimera is at least 1,000-fold more active than its dimeric CD4–IgG counterpart in syncytium inhibition assays and that effector functions, such as the binding of Fc receptors and the first component of the complement cascade (C1q), are retained. Similar chimaeric molecules, combining CD4 with human IgG were recently described by Capon *et al.*¹⁰, but these included the CH1 domain and did not bind C1q. Deletion of the CH1 domain may allow the association and secretion of heavy chains in the absence of light chains¹¹, and we suggest that the basic design of our constructs may be generally and usefully applied.

We have previously produced chimaeric proteins consisting of different domains of CD4 and immunoglobulin constant region of mouse κ (M κ). If we denote the four suggested extracellular domains of CD4 protein starting at N-terminus by 1, 2, 3 and 4 (ref. 12), the following proteins were generated: 1·C κ , 1·2·C κ , 1·2·3·C κ and 1·2·3·4·C κ (ref. 13). All these

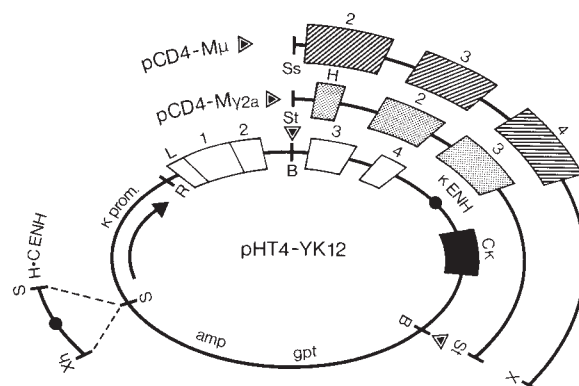
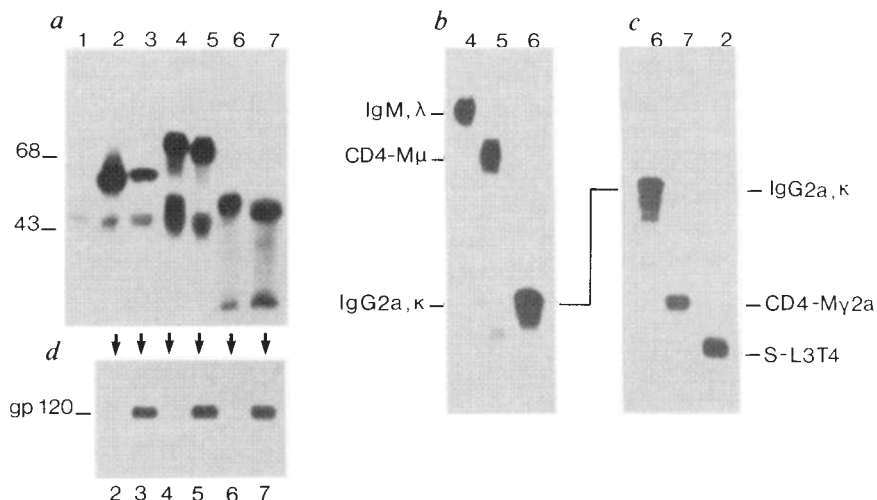


FIG. 1 Immunoglobulin-CD4 chimaeric constructs. The plasmid pHT4.YK12 encodes all four extracellular domains of CD4 linked to the mouse Ig κ constant regions. CD4–M μ and pCD4–M γ 2a encode the first two N-terminal CD4 domains linked to mouse Ig μ (CH2, CH3, CH4) and mouse Ig γ 2a (Hinge, C_{H2}, C_{H3}) constant regions, respectively. Numbers above the boxes refer to exons coding for different domains. ENH=Enhancer, L=Leader, H=Hinge, B=Bam HI, R=Eco RI, S=Sal I, Ss=Sst I, St=Stu I, X=Xba I and Xh=Xho I. **METHODS.** The CD4– κ expression vector pHT4-Y1 (ref. 9) was modified by replacing the immunoglobulin heavy chain promoter by a more efficient immunoglobulin κ promoter derived from the vector pKm 1 (ref. 32). The κ promoter was first subcloned into an intermediate vector PUC 18 as a Bgl II/Sal I fragment, then transferred as a 2.2 kilobase (kb) fragment [Hind III(blunt)/Eco RI] into pHT4-yi [Xba I(blunt)/Eco RI], to generate pHT4-YK12. The Bam HI fragment of pHT4-YK12 (encoding domains 3 and 4 of CD4 and C κ) was then replaced by either a 3.5 kb Sdt/Xba I fragment containing C_{H2}, C_{H3} and C_{H4} constant region exons of mouse Ig μ or a 3.0 kb Stu I fragment encoding the hinge, C_{H2} and C_{H3} constant region exons of mouse Ig γ 2a. All fragments were blunt-ended before ligation into the vector. The constructs pCD4–M μ and pCD4–M γ 2a were completed by inserting a heavy chain enhancer as a Sal I/Xho I fragment into the unique Sal I site 5' of the promoter.

molecules retained their gp120 binding activity, suggesting that the first V κ -like domain is sufficient. We first attempted to produce secreted CD4-immunoglobulin chimaeras with specificity against gp120 by generating double transfectants producing 1·C κ and normal immunoglobulin heavy chains. This approach failed, however, and we could not detect assembly into complete molecules, even though a number of heavy chains with different variable regions were tested.

FIG. 2 Characterization of the chimaeric molecules. Stable transformants were obtained by transfecting CD4 constructs into X63-0 myeloma cells by protoplast fusion. *a–c*, Western blot analysis of immunoprecipitated chimaeric or natural immunoglobulin molecules from cell culture supernatants after 10% PAGE run under reducing conditions (*a*) or after 3–7% gradient PAGE (*b*) or 7% PAGE (*c*) run under non-reducing conditions. Non-transfected X63-0 (1), soluble murine CD4, L3T4 (ref. 9) (2), HT4-YK12 (3), B1.8 (IgM, λ) (4), CD4–M μ (5), UPC10 (IgG2a, κ) (6), CD4–M γ 2a (7). Samples 1–3 were immunoprecipitated with rabbit anti-mouse κ , samples 4 and 5 with rabbit anti-mouse IgM, samples 6 and 7 with protein A. All antibodies and protein A were immobilized on Sepharose 4B. The results were visualized with a mixture of rabbit anti-mouse Ig κ , μ and γ antibodies in a first step, followed by radioactive donkey-anti-rabbit immunoglobulin. Standard molecular weight markers are shown on the left (in thousands). The background bands in *a* (1–5), of relative molecular mass ~45,000 are due to rabbit IgG released from the Sepharose when the samples were prepared for electrophoresis. *d*, Co-immunoprecipitation under identical conditions as those in *a–c*, but in the presence of metabolically labelled gp120 from the supernatant of HIV-1-infected H9 cells, was followed by PAGE and fluorography. **METHODS.** Four million H9/HTLV-III cells³³ were pulse-labelled as previously



described³⁴ with [³⁵S]cysteine for 4 h. The cells were then transferred into 4 ml of fresh medium and incubated for a further 14 h. After the chase the cells were quickly removed by pelleting at 400g and the supernatant was subjected to 150,000g sedimentation for 1 h. Cleared supernatant (50 μ l) was then incubated with the indicated culture fluids for 14 h at 4 °C and the immunoprecipitations were performed as described above.

The successful approach relied on two observations. First, $1 \cdot 2 \cdot C\kappa$ and $1 \cdot 2 \cdot 3 \cdot 4 \cdot C\kappa$ were secreted, whereas $1 \cdot C\kappa$ and $1 \cdot 2 \cdot 3 \cdot C\kappa$ were not, suggesting to us that CD4 domains associate pairwise into two stable units consisting of $1 \cdot 2$ and $3 \cdot 4$ domains respectively. Second, the analysis of human heavy-chain disease proteins has shown that heavy chains of different subclasses can be secreted without light chains when the first constant region domain is deleted¹¹. We therefore constructed plasmids in which the exons encoding the first two N-terminal domains of CD4 were linked to all but the V_H1 and C_H1 domains of either the mouse μ heavy chain (CD4-M μ) or the mouse $\gamma 2a$ heavy chain (CD4-M $\gamma 2a$) (see Fig. 1).

Both CD4-M μ and CD4-M $\gamma 2a$ were found in the culture supernatants at levels of $1\text{--}5 \mu\text{g ml}^{-1}$ after the corresponding constructs were introduced into a myeloma cell line X63-0 (Fig. 2). Immunoprecipitations of the secreted proteins with relevant antibodies or protein A and subsequent western blot analyses showed that the molecules produced had the expected apparent molecular weights (Fig. 2). A similar analysis under non-reducing conditions indicated that CD4-M μ was most probably secreted as a pentamer, whereas CD4-M $\gamma 2a$ formed dimers, consistent with the fact that CD4-M $\gamma 2a$ bound to protein A (ref. 14) (Fig. 2b, c). Both CD4-M μ and CD4-M $\gamma 2a$, as well as the previously produced CD4-M κ , were able to bind to HIV gp120 (Fig. 2d). Because recombinant gp120 was not available, we used metabolically labelled preparations of gp120 derived from the supernatants of HIV-infected (H9) cell cultures in our co-immunoprecipitation tests.

Importantly, the effector functions of normal immunoglobulin molecules, such as binding to Fc γ receptors and Clq were kept intact in the hybrid molecules (Fig. 3). This suggests that removal of the CH1 domain does not create major structural alterations

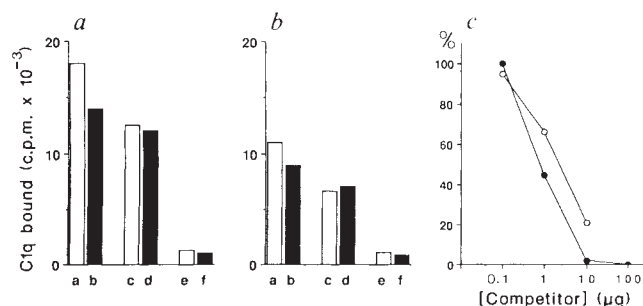


FIG. 3 Characterization of binding properties of CD4-immunoglobulin molecules. a, b, Clq binding assay. Microtitration plates were coated with purified CD4-immunoglobulin and natural control proteins of appropriate subclasses at the concentrations of $10 \mu\text{g ml}^{-1}$ (a) and $1 \mu\text{g ml}^{-1}$ (b). The direct binding of ^{125}I -labelled Clq was then measured. a, CD4-M $\gamma 2a$; b, OKT3 (IgG2a, K); c, CD4-M μ ; d, TEPC 183 (IgM, K); e, CD4-M κ ; f, BSA. c, Binding of CD4-immunoglobulin molecules to Fc γ receptors on the mouse macrophage cell line M29. Competition between ^{125}I -labelled CD4-M $\gamma 2a$ and the indicated amounts of CD4-M $\gamma 2a$ (○) and OKT3 (●) (IgG2a) proteins. METHODS. a and b, Polyvinyl chloride microtitration plates were coated with $100 \mu\text{l}$ of test and control proteins in saline at concentrations of $10 \mu\text{g ml}^{-1}$ (a) and $1 \mu\text{g ml}^{-1}$ (b). After overnight incubation the plates were blocked with 1% BSA solution and binding of ^{125}I -labelled human Clq ($\sim 2 \text{ ng}$, 50,000 c.p.m.) was measured after incubation for 6 h at room temperature. Radiolabelling of Clq (a gift from Dr A. Erdei) was carried out by the iodogen method, according to the recommendations of the manufacturer (PIERCE) c, Fc γ receptor binding was assayed by incubating 2×10^6 M29 mouse macrophage cells (a gift from Dr G. Stockinger) with ^{125}I -labelled CD4-M $\gamma 2a$ (200 ng), plus competitor proteins in $100 \mu\text{l}$ medium containing 5% fetal calf serum for 1 h at 4°C . After incubation the samples were centrifuged through a 200 μl cushion of fetal calf serum and the radioactivity in the pellets was measured. The radioactivity obtained after adding 500-fold excess of unlabelled IgG2a (OKT3) was assumed to be due to nonspecific binding and was subtracted before calculating the percentage inhibitions shown. Radiolabelling was performed as described above.

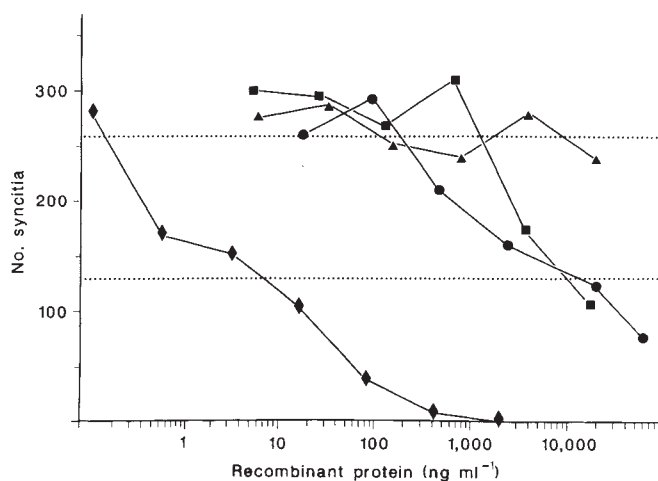


FIG. 4 Inhibition of syncytium formation by different recombinant proteins. The number of syncytia is plotted against the final concentrations of the recombinant proteins (●) CD4-M μ ; (■) CD4-M $\gamma 2a$; (●) CD4-M κ ; (▲) L3T4-M κ (ref. 9). The dotted lines indicate the number of syncytia in the absence of any inhibitory proteins and the number at 50% inhibition.

METHODS. A pretitrated amount of HIV-1-HAN was incubated at room temperature with serially diluted recombinant proteins for 30 min. Thereafter, $25 \mu\text{l}$ of each mixture was transferred in triplicate into the wells of a 96-well plate which contained 25,000 MT-2 cells³⁵ per well in $50 \mu\text{l}$ of medium. After 3 days culture, $100 \mu\text{l}$ of fresh medium was added per well and after 5 days the syncytia were counted. The sums of syncytia of the triplicates were used for inhibition curves. HIV-1-HAN has been isolated from the PBL of an AIDS patient. Partially determined nucleotide sequence of HIV-1-HAN shows about 90% sequence homology to HTLV-III_B (U. Sauer-mann and J. Mous, unpublished observation). Culture supernatants of MT-2 or Jurkat cells infected with HIV-1-HAN contained ten times more syncytium-forming capacity than similar culture supernatants of H9/HTLV-III_B cells.

in the regions of the CH2 domain responsible for Clq and Fc γ receptor binding.^{15,16}

To assay the biological activity of these molecules, we tested them in the syncytium inhibition assay^{17,18} (Fig. 4). CD4-M μ was by far the best inhibitor of syncytium formation: 50% inhibition was obtained at a concentration of 10 ng ml^{-1} which was about 1,000-fold less than the concentration ($\sim 10 \mu\text{g ml}^{-1}$) of CD4-M $\gamma 2a$ and CD4-M κ proteins needed for the same effect. Complete abolition of syncytia formation was possible with CD4-M μ at concentrations of about $1\text{--}2 \mu\text{g ml}^{-1}$. The CD4-M κ protein exists as a noncovalently-associated dimer (data not shown), possibly due to the $C\kappa$ portion of the molecules. Thus it seems that the effectiveness of these molecules increases as a function of their valence. Truly monovalent CD4, which we do not have, has not been compared directly with the dimeric forms of recombinant CD4 molecules (CD4-M $\gamma 2a$ and CD4-M κ) in this particular assay.

Soluble CD4 provides the optimal specificity for neutralization of the HIV-1 for many reasons. First, the strength of the interaction of the CD4 and gp120 is very high, of the order of 10^{-9} M (refs 5, 19). Second, the genetic variants of HIV-1 (refs 20–23) which emerge frequently during the infection must retain their CD4-binding properties to maintain their infectivity. Third, the immunity against gp120 acquired during the infection can have serious deleterious effects on the immune system: free gp120 which is shed from the virus^{24,25} can be trapped specifically on the CD4-positive cells and in this way the non-infected cells can become targets of various forms of anti-gp120 immune attacks^{26–29}. Passive immunity based on CD4 specificity would avoid this bystander destruction because it would discriminate between gp120 molecules which are already bound on cell-surface CD4 and those molecules which are produced by infected cells.

Although the human CD4-immunoglobulin chimera reported by Capon *et al.*¹⁰ was secreted, they found that a similar hybrid protein, based on the mouse γ_1 heavy chain, was retained intracellularly. We have also noticed that hybrid molecules containing the C_H1 domain, for example CD4-IgM chimaeras, are not secreted (unpublished observation) and we suspect that in the absence of immunoglobulin light chains, the hydrophobic face of the C_H1 domain interacts strongly with the heavy chain

binding protein, thus preventing secretion^{30,31}.

We believe that hybrid proteins which combine the specificity of CD4 with the multivalency and effector functions of different immunoglobulin subclasses could provide a realistic approach to AIDS therapy. We also think that our approach to designing hybrid immunoglobulin molecules could be applied more generally for building novel immunoglobulin molecules. □

Received 27 February; accepted 27 March 1989.

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ACKNOWLEDGEMENTS. We would like to thank Ludwina Ahlborn, Filippo Oliveri, John Hatton and Reinhard Schulze for technical assistance, Nicole Schoepflin for preparing the manuscript, Hans Peter Stahlberger for the graphic and Drs Alan Tunnaciff and Richard Scheuerman for critical reading. We would also like to thank Dr Ulrike Sauermaun, German Primate Center for providing HIV-1-HAN and Dr Jan Mous for discussions. The Basel Institute for Immunology was founded and is supported by F. Hoffmann La-Roche Ltd Co., Basel, Switzerland.

Activation of HIV gene expression during monocyte differentiation by induction of NF- κ B

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THE latent period of AIDS is influenced by factors which activate human immunodeficiency virus (HIV) replication in different cell types. Although monocytic cells may provide a reservoir for virus production *in vivo*^{1–8}, their regulation of HIV transcription has not been defined. We now report that HIV gene expression in the monocyte lineage is regulated by NF- κ B, the same transcription factor known to stimulate the HIV enhancer in activated T cells⁹; however, control of NF- κ B and HIV in monocytes differs from that observed in T cells. NF- κ B-binding activity appears during the transition from promonocyte to monocyte in U937 cells induced to differentiate *in vitro* and is present constitutively in mature monocytes and macrophages. In a chronically infected promonocytic cell, U1, differentiation is associated with HIV-1 replication as well as NF- κ B binding activity. These findings suggest that NF- κ B binding activity is developmentally regulated in the monocyte lineage, and that it provides one signal for HIV activation in these cells.

We transfected monocytic cell lines from progressive stages of differentiation with a plasmid containing the HIV enhancer linked to the chloroamphenicol acetyltransferase (CAT) gene. Twenty-four hours after transfection, cells were incubated in medium alone or in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Expression of the HIV enhancer was induced by TPA in two immature monocyte leukaemia lines, a human granulocyte-macrophage leukaemia, HL-60, and the human promonocytic line, U937 (Fig. 1a). TPA treatment did not augment CAT expression in the mature macrophage leukaemic cells, THP-1, P388D1, or PU5-1.8, which showed higher basal activity (Fig. 1b; note scale changes).

Using a mutant HIV-CAT plasmid containing alterations in both κ B sites⁹, we showed that induction of HIV-CAT expression in the immature lines, HL-60 and U937 (Fig. 1a), and constitutive expression in the mature lines was dependent on the κ B sites (Fig. 1b). This suggested that NF- κ B is present in the induced progenitors and in the mature cells, and we therefore looked for NF- κ B binding activity in nuclear extracts from these cell lines. NF- κ B binding activity in the immature lines, HL-60 and U937, was induced by TPA, whereas in the mature macrophage lines, THP-1, PU5-1.8, and P388D1, it was constitutively expressed (Fig. 2a). We then determined whether NF- κ B binding activity is present in normal human monocytes and/or macrophages. Nuclear extracts were prepared from human peripheral blood monocytes or adherent mononuclear cells, and NF- κ B binding activity was found in both cell types as well as in mouse peritoneal macrophages (Fig. 2b). NF- κ B binding is therefore constitutively active in normal and neoplastic mature mononuclear phagocytes, including blood monocytes and adherent macrophages.

Treatment of immature monocytes with TPA, or the water-soluble phorbol-12, 13-dibutyrate¹⁰ (PDB) (which is more easily removed from cells) causes differentiation into mature monocytes and macrophages, as judged by changes in cell growth, morphology, surface glycoproteins, and phagocytic function (Fig. 3, see also refs 11–15). HL-60 cells treated with PDB acquired characteristics of mature macrophages, displaying growth arrest, increased phagocytosis, adherence, FcR and Mo 1 expression. At the same time, these cells began to express NF- κ B binding activity which persisted even two days after

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removal of PDB (Fig. 3a). U937 cells matured similarly and also began to express NF- κ B binding constitutively, both in the presence of PDB or tumour necrosis factor- α (TNF- α) (Fig. 3b), another known inducer of NF- κ B (ref. 16) and monocyte differentiation (Fig. 3b, see also refs 14, 15). In contrast to HL-60 cells, U937 cells did not become adherent after incubation with PDB or TNF- α . These findings, together with the detection of NF- κ B in circulating monocytes (Fig. 2b), suggests that NF- κ B induction is associated with the transition from promonocyte to monocyte.

To determine whether monocyte differentiation and NF- κ B induction affect production of intact virus, the latently infected U1 promonocytic line¹⁷ was incubated with PDB or TNF- α . U1 cells exposed to these agents demonstrated morphological,

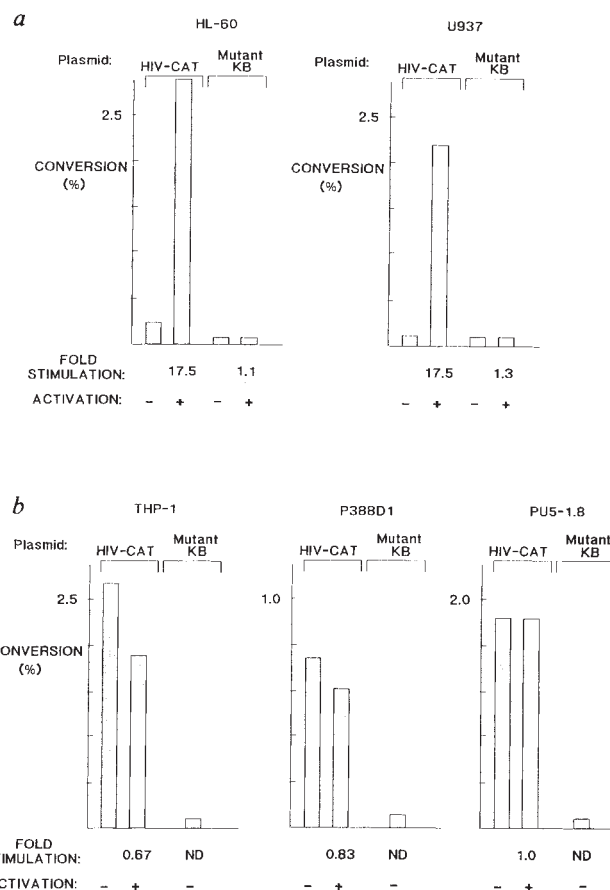
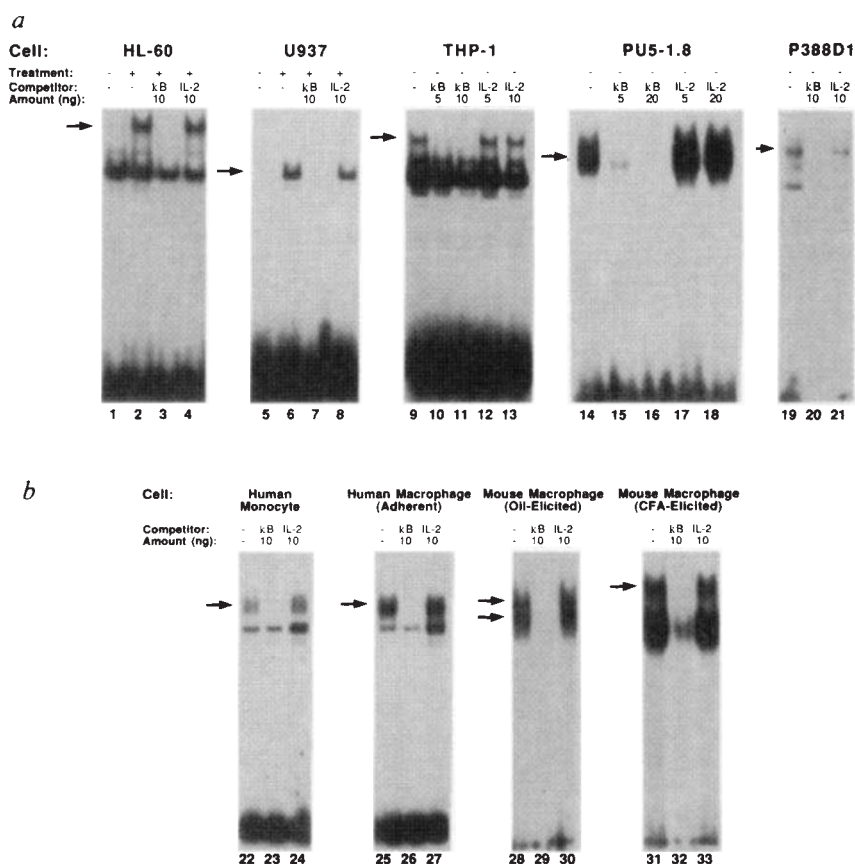


FIG. 1 Activation of HIV-CAT expression in immature cells of the monocytic lineage, constitutive activity in mature cells, and dependence on κ B sites. The indicated monocytic leukaemia lines (10^7) were transfected with an HIV-CAT or a mutant plasmid (10μ g) altered in both κ B sites using DEAE-dextran⁹. Twenty-four hours after transfection, an aliquot of cells was maintained in medium alone (-) or incubated for a further 20 h in the presence of 20 nM TPA (+) (Sigma). The monocytic leukaemia cells were representative of progressive stages of maturity and included (a) immature cells HL-60 (human granulocyte-macrophage progenitor) or U937 (human promonocyte), and (b) mature cells THP-1 (human macrophage), P388D1 (mouse macrophage), or PU5-1.8 (mouse macrophage) leukaemia lines. **METHODS.** Cells were transfected as previously described⁹, except that cells were treated with 10 μ M chloroquine for 30 min after transfection. Cell extracts were prepared 44 h after transfection, protein concentrations normalized and conversion of chloramphenicol to its acetylated forms assayed by standard methods²⁹. Results are representative of at least two independent transfections and the standard deviations of each determination was <10%. Per cent conversion of [¹⁴C]chloramphenicol to its acetylated forms are shown.

FIG. 2 NF- κ B binding is induced by TPA in immature monocytic lines and found constitutively in mature macrophage lines. Nuclear extracts were prepared from cells treated in medium alone (-) or for 2 h in the presence of 40 nM TPA (+) and analysed using the electrophoretic mobility shift assay with a κ B probe³⁰. **a**, Nuclear extracts from transformed leukaemic lines: HL-60 leukaemia cells (lanes 1-4), U937 promonocytic cells (lanes 5-8), the THP-1 human myelomonocytic leukaemia line (lane 9-13), the PU5-1.8 mouse macrophage leukaemia line (lane 14-18) or the P388D1 mouse macrophage line (lane 19-21). **b**, Nuclear extracts from normal cells: human monocytes (lanes 22-24), human adherent macrophages (lanes 25-27), mouse mineral oil-elicited adherent macrophages (lanes 28-30) or mouse complete Freund's adjuvant-elicited adherent macrophages (lanes 31-33). Adherent cells were $\geq 95\%$ pure and mononuclear cells $\sim 80\%$ enriched, (as confirmed by phagocytosis, histochemical analysis, and Mo 1 immunofluorescence). THP-1 cells expressed low levels of Fc receptor and low levels of Mo 1 antigen, which increased in response to TPA, together with adherence. TPA also induced rapid secretion of IL-1 and TNF- α , suggesting a mature phenotype. Extracts were incubated with a κ B probe alone or in the presence of the indicated amounts of double-stranded oligonucleotide competitor containing the κ B site³⁰ or an unrelated IL-2 promoter fragment³¹. Arrows denote specific inducible complexes competed by double-stranded κ B oligonucleotide.

METHODS. The electrophoretic mobility shift assay was performed using a κ B probe as previously described³⁰, with 10 μ g nuclear extract in the presence of 1 μ g dIdC. Extracts were prepared for analysis from these cells by a rapid method previously described¹⁶.



functional and cell-surface characteristics of differentiation similar to those seen in U937 cells^{17,18}, and NF- κ B binding was induced (Fig. 4a, lanes 2 and 3). At the same time, reverse transcriptase and p24 antigen levels increased dramatically (Fig. 4b, c). Both NF- κ B and HIV production persisted even 24–48 h after the stimulus was removed (Fig. 4).

Transient exposure of immature lines to PDB (<2 h) did not lead to constitutive expression of NF- κ B binding, and had no effect on cell morphology (data not shown). Once monocyte differentiation occurred, however, NF- κ B binding activity remained, as it does in normal monocytes (Fig. 2b). Constitutive NF- κ B binding activity, found previously only in mature B-cell leukaemia lines¹⁹, is therefore associated with mature cells of the monocyte lineage. NF- κ B binding is also inducible in different cells by several agents, including TPA, TNF- α , and IL-1 (refs 9, 16, 20).

In these experiments, NF- κ B binding was induced when

growth was inhibited, raising the possibility that it is associated with growth arrest. TNF- α , which also induces macrophage differentiation and growth arrest, (Fig. 3b; refs 14, 15) stimulates NF- κ B binding activity¹⁶. TNF- α also activates HIV expression in T cells^{16,21}. This cytokine is elevated in the blood of AIDS patients²², and may be important in the activation of HIV *in vivo*.

The HIV enhancer can be activated through κ B-independent mechanisms²³ and contains additional transcription factor binding sites^{24,25}. Our data show that NF- κ B contributes to the activation of HIV in monocytes, but it is not the sole activator of virus in these cells. Granulocyte-macrophage colony-stimulating factor, GM-CSF, which does not induce NF- κ B binding¹⁶, activates HIV expression in mature macrophages²⁶ which already contain NF- κ B (Fig. 2b), and, in combination with TNF- α , in the U1 cell line (data not shown). These findings imply that at least two signals modulate HIV production in

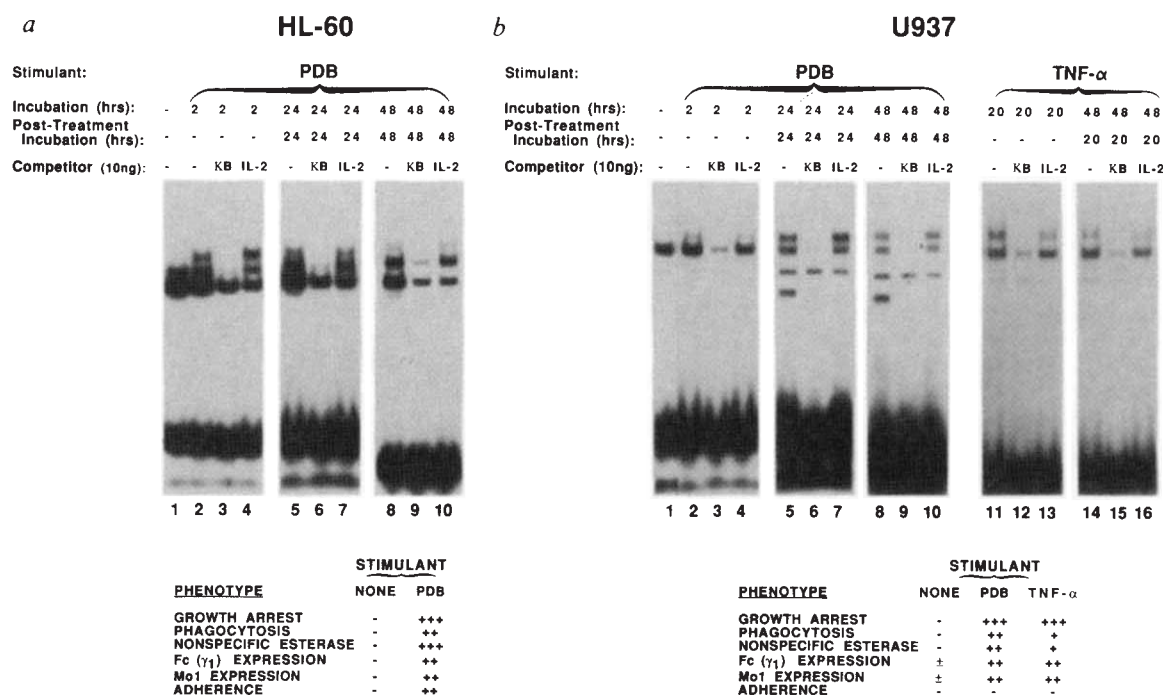
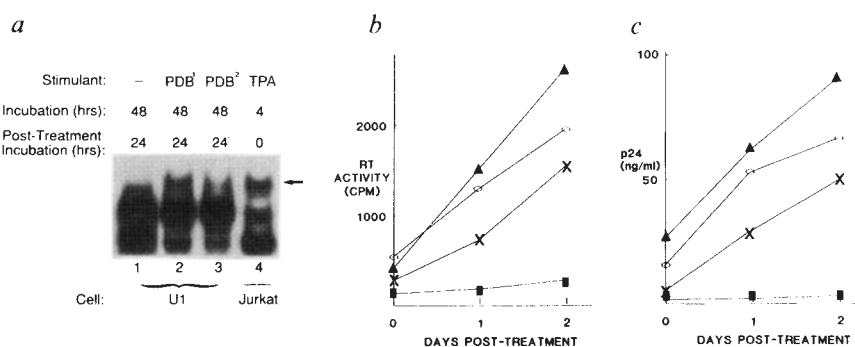


FIG. 3 Induction of NF- κ B binding activity during macrophage differentiation. Nuclear extracts were prepared from HL-60 (a) or U937 (b) leukaemia lines after the indicated treatments and analysed using the electrophoretic mobility shift assay. HL-60 cells were maintained in medium alone (—) (a, lane 1) or incubated with 20 nM PDB (+) for the indicated time, followed by three washes, and incubated for the additional time indicated in the absence of PDB (a, lanes 2–10). U937 cells were similarly incubated alone (b, lane 1),

with 20 nM PDB (b, lanes 2–10), or with 50 ng ml⁻¹ TNF- α (b, lanes 11–16). Specificity of binding was determined by competition with the indicated double-stranded oligonucleotide competitors. Arrows denote specific inducible complexes competed by double-stranded κ B oligonucleotide. Cell lines were analysed for the presence of the Mo1 antigen or FcR using a fluorescence-activated cell sorter (refs 32, 33), and analysed by standard methods³⁴ for non-specific esterase, phagocytosis, and growth arrest.

FIG. 4 Increased constitutive expression of NF- κ B and HIV in a chronically-infected promonocytic line following cellular differentiation. a, NF- κ B binding activity was analysed as before. Cellular extracts were prepared from U1 cells incubated in medium alone (lane 1), in the presence of 2 nM PDB (ref. 1) (lane 2) or 20 nM PDB (ref. 2) (lane 3) for the indicated time, followed by washing and further incubation in medium alone for an additional 24 h. A positive control using extract from TPA stimulated Jurkat cells is included (lane 4). Arrows denote specific inducible complex competed by double-stranded κ B oligonucleotide. b and c, Reverse transcriptase (RT) activity (b) was determined¹⁷, and p24 antigen levels (c) quantitated (Coulter Immunology) in supernatants from U1 cells incubated in medium alone (□) or in the presence of 2 nM PDB (▲), 20 nM PDB (○) or 1,000 U ml⁻¹ TNF- α (x) for 48 h, followed by washing, and incubation for an additional



24 or 48 h. Reverse transcriptase values for these groups before the 48 h wash were 101, 379, 526, and 250 c.p.m. respectively.

mononuclear cells. Because hematopoietic stem cells can be infected by HIV^{27,28}, we suggest that HIV might be induced in latently infected promonocytic stem cells by NF- κ B activated during monocyte differentiation, and additionally by signals such as GM-CSF, which may act through an independent pathway. □

Received 19 December 1988; accepted 20 March 1989.

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ACKNOWLEDGEMENTS. We thank Bernice Bishop and Roberta Call for typing the manuscript, and Victor Perez for assistance in the U1 induction experiments. This work was supported in part by the Wellcome Trust (G.E.G.) and the National Institutes of Health (G.J.N. and S.L.K.).

Transfer of a β -turn structure to a new protein context

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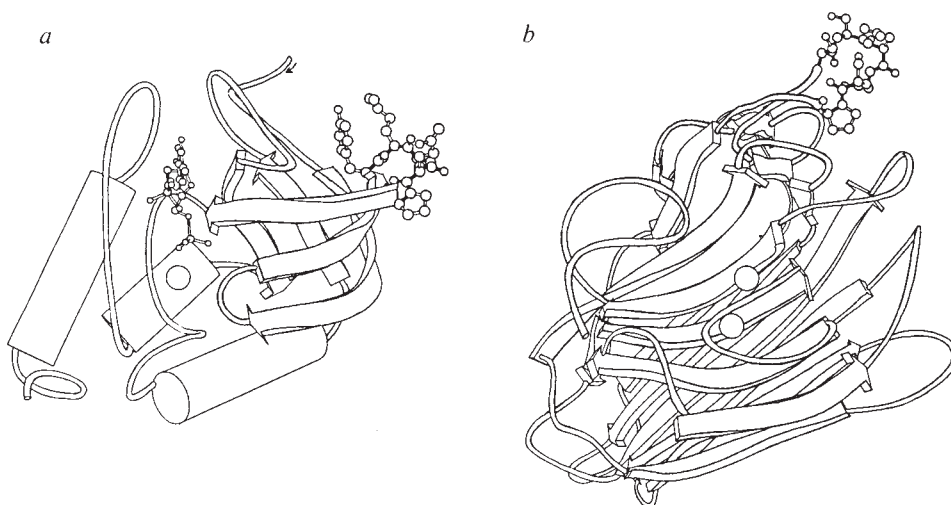
FOUR-RESIDUE β -turns and larger loop structures represent a significant fraction of globular protein surfaces and play an important role in determining the conformation and specificity of enzyme active sites and antibody-combining sites^{1,2}. Turns are an attractive starting point to develop protein design methods, as they involve

a small number of consecutive residues, adopt a limited number of defined conformations and are minimally constrained by packing interactions with the remainder of the protein. The ability to substitute one β -turn geometry for another will extend protein engineering beyond the redecoration of fixed backbone conformations to include local restructuring and the repositioning of surface side chains. To determine the feasibility and to examine the effect of such a structural modification on the fold and thermodynamic stability of a globular protein, we have substituted a five-residue turn sequence from concanavalin A for a type I' β -turn in staphylococcal nuclease. The resulting hybrid protein is folded and has full nuclease enzymatic activity but reduced thermodynamic stability. The crystal structure of the hybrid protein reveals that the guest turn sequence retains the conformation of the parent concanavalin A structure when substituted in the nuclease host.

An examination of turn sites in structurally related proteins illustrates that considerable variation is possible in sequence length and backbone conformation³. An analysis of protein structures has also identified correlations between β -turn sequence and conformation^{4,5}, although the local sequence alone does not uniquely determine the fold of short protein

FIG. 1 *a*, The overall fold of nuclease represented as cylinders (α -helices) and arrows (β -strands). The β -turn region, residues 27-31 (Tyr-Lys-Gly-Gln-Pro), is shown in atomic detail in the upper right. A nuclease structure refined in our laboratory as well as a refinement of the Ca^{2+} pdTp complex of nuclease²² show turn residues 27-30 in a type I' β -turn conformation differing from the 2SNS nuclease structure^{23,24} in the Brookhaven Protein Data Bank²⁵. The active site is indicated by bound pdTp nucleotide (small atoms) and Ca^{2+} (large circle). *b*, The overall fold of a concanavalin A monomer unit with arrows representing the β -strands. Turn residues 160-165 (Ser-Ser-Asn-Gly-Ser-Pro) are shown in atomic detail. Residues 160-163 form a type I β -turn. Two structures of concanavalin A have been determined at high resolution^{11,12} (2CNA,3CNA). In both cases weak electron density was observed over turn residues 160-165, leading to slightly different models. The primary discrepancy between the two is the orientation of the peptide unit adjacent to the β -turn joining Gly 163 and Ser 164. The bound Ca^{2+} and Mn^{2+} ions are represented as large circles. Both *a* and *b* were drawn with the aid of the program ARPLOT²⁶.

METHODS. The nuclease gene was subcloned into M13mp18. Two rounds



of primer-directed mutagenesis introduced *Hind*III and *Bgl*II sites flanking the β -turn sequence at codons 27-31 and removing a *Hind*III site within the gene (J.F.G. unpublished data). An oligonucleotide encoding the concanavalin A β -turn sequence was synthesized and subcloned into the vector described above. The hybrid gene was subcloned into the pAS1 expression construct and protein prepared as described²⁷.

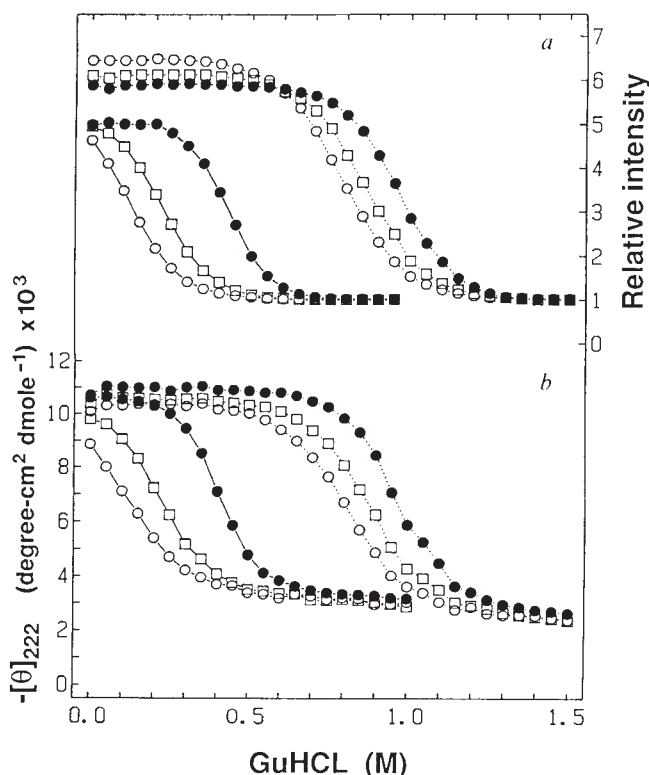


FIG. 2 The sensitivity of nuclease (dotted lines) and the hybrid protein (solid lines) to guanidine hydrochloride (GuHCl) denaturation. Open circles, protein in buffer alone; open squares, buffer plus 10 mM CaCl_2 ; filled circles, buffer, 10 mM CaCl_2 and 50 μM pdTp inhibitor. *a*, Fluorescence from the single tryptophan at residue 140. Excitation wavelength 300 nm, emission monitored at 340 nm using an SLM Model 8000 fluorimeter. *b*, Circular dichroism (molar ellipticity at 222 nm) monitored using a AVIV Model 60DS instrument. All measurements were made in 1 cm-pathlength cuvettes at 25 °C with a protein concentration of 6 μM (0.1 mg ml^{-1}), in a buffer of 100 mM Tris, 100 mM NaCl, pH 7.4. From the fluorescence data, the concentration of GuHCl which produced half unfolding (C_m), and the Gibbs free energy of denaturation extrapolated to zero denaturant concentration¹⁶ (ΔG_D) are as follows: nuclease in buffer alone, $C_m=0.78$ M, $\Delta G_D=4.6$ kcal mol^{-1} ; plus Ca^{2+} , $C_m=0.86$ M, $\Delta G_D=5.2$ kcal mol^{-1} ; Ca^{2+} plus pdTp, $C_m=0.96$ M, $\Delta G_D=6.3$ kcal mol^{-1} ; hybrid protein in buffer alone, $C_m=0.14$ M, $\Delta G_D=1.2$ kcal mol^{-1} ; plus Ca^{2+} , $C_m=0.24$ M, $\Delta G_D=1.9$ kcal mol^{-1} ; Ca^{2+} plus pdTp, $C_m=0.43$ M, $\Delta G_D=3.9$ kcal mol^{-1} .

segments^{6,7}. A search of conformational space for short peptide segments taken from known protein structures has proven successful in modelling the correct polypeptide backbone geometry when the ends of the segment are constrained to join the remaining structure⁸⁻¹⁰. These observations suggest that the conformation of a short peptide segment is determined by its sequence and the geometry of the flanking chain, indicating that β -turns may be transferred from one protein context to another as a structural cassette.

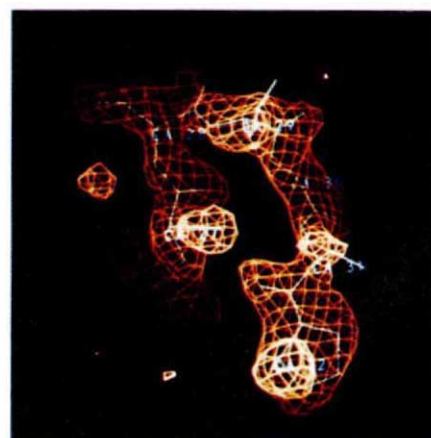
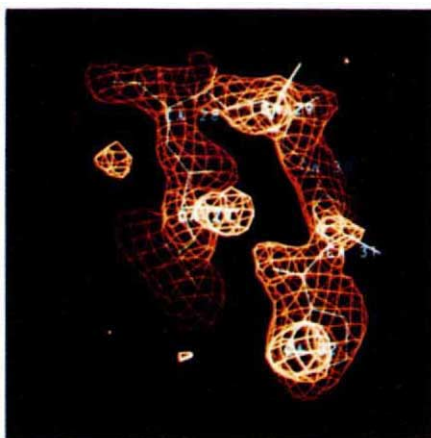
The nuclease host site is a type I' β -turn (residues 27-30)

which connects two antiparallel β -strands forming one side of a five-stranded β -barrel (Fig. 1*a*). A five-residue turn from concanavalin A^{11,12} (Fig. 1*b*) was selected for the guest turn sequence as it is possible to align the β -strands leading away from this turn with the nuclease β -strands (Fig. 4*a*). The five-residue turn consists of a type I β -turn adjacent to a G1 β -bulge^{13,14}. The modelling indicated that nuclease residues 27-31 (Tyr-Lys-Gly-Gln-Pro) could be replaced with concanavalin A residues 160-165 (Ser-Ser-Asn-Gly-Ser-Pro). This hybrid protein was constructed and expressed in *Escherichia coli* (Fig. 1).

The hybrid protein is folded and has full nuclease hydrolysis activity against saturating concentrations of heat-denatured DNA¹⁵ (25 °C, 25 mM Tris, 10 mM CaCl_2 , pH 8.8; data not shown). The stability of nuclease and the hybrid protein was determined by guanidine hydrochloride (GuHCl) denaturation, monitored by fluorescence and circular dichroism (Fig. 2). The half-unfolding point (C_m) of nuclease occurs at 0.78 M GuHCl with a free energy of denaturation¹⁶ (ΔG_D) of 4.6 kcal mol^{-1} . The unfolding transition shifts to a higher C_m with the addition of Ca^{2+} and deoxythymidine-3',5'-diphosphate (pdTp), a competitive inhibitor of the enzyme¹⁵ (Fig. 2). Although the stability of the hybrid protein is significantly reduced ($C_m=0.14$ M,

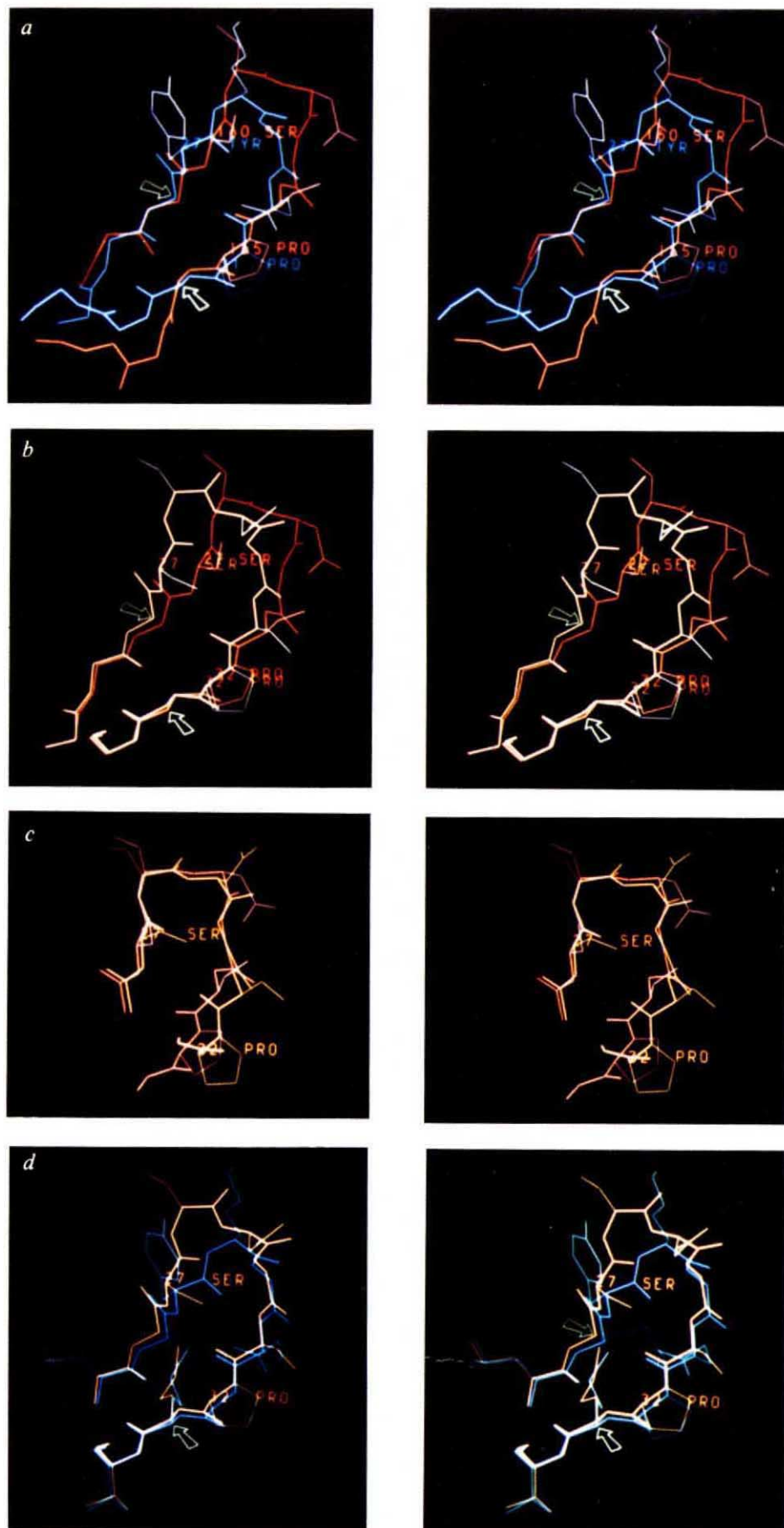
FIG. 3 Structure of the guest turn shown with a 1.8 Å $F_o - F_c$ residue-deleted electron density map, contoured at 3 σ (σ is the root-mean-square difference electron density in the unit cell). The concanavalin A residues 160-165 have been renumbered as hybrid protein residues 27-32. Density from a bound water molecule is seen to the left of the amide nitrogen of Ser 28. The six turn residues were omitted from the $F_o - F_c$ Fourier map calculation to remove the bias of phase information contributed by the guest turn model.

METHODS. Nuclease and the hybrid protein were crystallized out of low-salt buffer (10.5 mM potassium phosphate, pH 8.15) using 2-methyl-2,4-pentanediol as a precipitant²⁸ yielding bipyramidal crystals in space group $P4_1$. Unit cell dimensions were within 1% of values from the published structure²³. Diffraction data from a single crystal of the hybrid protein bound with Ca^{2+} and pdTp inhibitor ($a=b=48.50$ Å, $c=63.19$ Å), was collected to 1.8 Å resolution at 25 °C using a Xuong-Hamlin area detector system²⁹ at Yale University. 29,913 measurements of 13,418 unique reflections were collected, covering 93% of the possible reflections to 1.8 Å. The merging R -factor on intensity was 2.3%. With starting phases calculated from the published nuclease coordinates^{23,24}, the hybrid structure, including 84 bound water molecules, was refined to 1.8 Å resolution with alternating cycles of Hendrickson-Konnert refinement³⁰ and model building with the molecular



graphics program FRODO³¹ (final R -factor=16.6%, r.m.s. bond distance=0.017 Å, overall temperature factor=20.4 Å²). A local scale was applied to the observed structure factor amplitudes relative to the calculated structure factor amplitudes to correct for absorption and anisotropic diffraction³². The box size was 5 × 5 × 5, with any reflection with fewer than 40 observed reflections in the box eliminated from the data set. Diffraction data on nuclease crystals without bound ligand ($a=b=48.5$ Å, $c=63.5$ Å) were collected to 1.7 Å resolution at 25 °C using a Nicolet-Xentronics area detector system³³ at Yale University. Details of the refinement will be published elsewhere (final R -factor=16.0%, r.m.s. bond length=0.015 Å, overall temperature factor=22.4 Å²).

FIG. 4. *a*, Structure of the concanavalin A (3CNA) turn in red, superimposed on the nuclease turn in blue. The coordinates of the nuclease β -turn region were refined in our laboratory (see Fig. 3). The concanavalin A model is oriented to bring the β -strands below the turn in register with the nuclease β -strands. Junctions between the sequences, indicated by the arrows, were chosen where α -carbons were within 0.2 Å and the trajectory of the backbones were similar. This incorporates one additional residue in the turn with nuclease residues 27–31 (Tyr-Lys-Gly-Gln-Pro) replaced by concanavalin A residues 160–165 (Ser-Ser-Asn-Gly-Ser-Pro). The atoms of the concanavalin A turn as modelled do not contact neighbouring atoms of the nuclease host. *b*, The guest turn from the hybrid protein crystal structure in yellow, superimposed on the starting model of the guest turn in red. Arrows indicate the junctions between nuclease and concanavalin A sequences. The guest-turn residues have been numbered 27–32. The β -strands below the junctions of the hand-built starting model are taken from nuclease as illustrated above (Fig. 4*a*). *c*, The guest turn from the crystal structure of the hybrid protein in yellow, superimposed on the same sequence from the structure of concanavalin A (3CNA) in red. The superposition is a least-squares fit between the backbone atoms (N, C α , C, O) of β -turn residues 27–30 of the hybrid with backbone atoms of β -turn residues 160–163 of concanavalin A. The r.m.s. deviations of backbone atoms of the six guest turn residues with respect to the 2CNA and 3CNA turn structures are 0.93 Å and 0.84 Å, respectively (for the four β -turn residues the r.m.s. deviations are 0.87 Å and 0.62 Å, respectively). The r.m.s. deviation between the backbone atoms of the two concanavalin A structures is 0.92 Å for all six residues and 0.74 Å for the four β -turn residues. *d*, The guest turn from the crystal structure of the hybrid protein in yellow, superimposed on the nuclease turn in blue. Arrows indicate the junctions between nuclease and concanavalin A sequences. The side-chain atoms of residues below the arrows are included for comparison. For the two residues on either side of the turn site the r.m.s. deviation is 0.13 Å for the backbone atoms and 0.33 Å for all atoms. The r.m.s. deviation between nuclease and hybrid backbone atoms excluding the turn residues, a disordered loop (residues 40–54) and residues involved in nucleotide binding (residues 112–115) is 0.22 Å.



$\Delta G_D = 1.2 \text{ kcal mol}^{-1}$) the C_m increases with additions of Ca^{2+} and pdTp (Fig. 2). The presence of full enzymatic activity, together with the stabilization by Ca^{2+} and pdTp, indicate that the active site, and thus the tertiary structure, has not been altered by the turn substitution.

The crystal structure of the hybrid protein was determined and refined at 1.8 Å resolution. The guest turn structure and a 1.8 Å-resolution residue-deleted $F_o - F_c$ electron density map are shown in Fig. 3. The backbone conformation is unambiguous, with the first four residues in a type I β -turn conformation. Side-chain density is present for Ser 27, Ser 28 and Pro 32, whereas Asn 29 is disordered and Ser 31 has density indicative of two side-chain conformations, one of which is modelled. The average temperature factor for the backbone atoms of the guest turn is 35.1 Å^2 , as contrasted with 23.7 Å^2 for residues 27–31 of the nuclease structure. The elevated temperature factor of the guest turn is consistent with the weak electron density reported for the same sequence in both concanavalin A structures^{11,12}, indicating that the guest turn has retained a similar degree of flexibility following transfer to the nuclease host.

The difference in the backbone conformation of the guest turn relative to the starting model (Fig. 4b) is primarily a rotation of the guest turn as a unit, seen by its superposition on the concanavalin A structure (Fig. 4c). The first four residues (Ser-Ser-Asn-Gly), adopt a type I β -turn conformation, Gly 30 retains the left-handed helix geometry of the G1 β -bulge. The pattern of backbone hydrogen bonding of the concanavalin A structure has been preserved in the guest turn structure. Because of the flexibility of the guest turn and the two concanavalin A structures, it is difficult to compare the details of side-chain orientation. Of the four guest turn residues with variable side-chain geometry, only the first two (Ser 27, Ser 28) are clearly defined in the electron density, with Ser 28 matching both concanavalin A structures.

The path of the guest-turn backbone closely follows the original nuclease-turn structure over the C-terminal end of the guest-turn sequence bulging out at the top of the turn (Fig. 4d). Residues adjacent in primary sequence at the base of the turn are unaffected by the guest turn. One change in the host structure is the side chain of Glu 10, which hydrogen bonds to the hydroxyl of Tyr 27 in nuclease, but shifts 2.5 Å in towards Ser 27 in the hybrid structure. In addition, the side chains of Phe 34 and Phe 76, buried below Tyr 27 in nuclease, become partially exposed to solvent in the hybrid structure. The exposure of these hydrophobic residues may explain the reduced stability of the hybrid protein. A point mutation of nuclease, replacing Tyr 27 with Ser, leads to a severe drop in enzymatic activity on indicator plates¹⁷, presumably as a result of a decrease in thermodynamic stability¹⁸.

The local backbone conformation of the hybrid protein and the number and orientation of side-chain positions have been altered (Fig. 4d). One possible determinant of both the type I' β -turn of nuclease and the five-residue guest turn conformation is the presence of Gly at residues 29 and 30, respectively, to permit the left-handed helix geometry¹³. The other turn positions could tolerate amino-acid substitutions to confer novel function. Engineering alternative local backbone conformations in combination with the generation of side-chain substitutions will add a new dimension to future protein design strategies. Applications include the modification of enzyme function and antibody recognition, in addition to the introduction of structural tags recognizable by the immune system^{19,20} and other processes of protein recognition²¹.

In this experimental example, the concanavalin A turn-conformation and the nuclease host tertiary structure are preserved on formation of the hybrid protein. The two structures are annealed through local backbone changes at the junction. Modest conformational changes occur in nuclease where side-chain contacts have been altered. In this instance the conformation of the guest turn was determined by the turn sequence and

the geometry of the host site, suggesting that protein engineering can be approached using β -turns as structural cassettes. □

Received 9 November 1988; accepted 14 March 1989.

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ACKNOWLEDGEMENTS. We thank Professor L. Stryer for use of the fluorimeter, Professor R. L. Baldwin for use of the circular dichroism instrument, and Dr P. J. Bjorkman for assistance with crystallographic programs. Dr J. B. Rothbard is gratefully acknowledged for many helpful discussions and for his assistance in the early molecular modelling studies and gene construction. This work was supported by grants to R.O.F. from the Weingart Foundation and the NIH. T.R.H. and M.A.G. received support from predoctoral training grants from the Markey Foundation and NIH.

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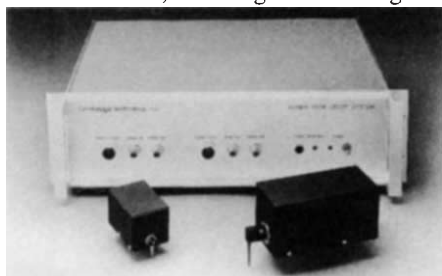
Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

Prelude to summer products

This week's issue spotlights a set of levers for muscle research, thermal cyclers for enzymatic DNA amplification reactions, and an oven for cooking up high-temperature superconductors.

BECKMAN Instruments unveiled its **capillary electrophoresis** instrument last month at the First International Symposium on High Performance Capillary Electrophoresis, joining the market with Bio-Rad, Applied Biosystems and Dionex (*Reader Service No. 100*). Beckman's P/ACE 2000 system comes with a built-in on-column detector, but its modular design will allow it to accommodate future detectors as the technology advances. Each capillary within the P/ACE system is housed in a temperature-controlled cartridge, and buffer and sample vials are sealed to eliminate the risk of evaporation. The P/ACE 2000 is available with manual or IBM computer control. Beckman Instruments is now taking reservations for the P/ACE 2000 system.

Cambridge Technology has a series of levers for measuring the dynamic physical properties of all types of muscle and connective tissue (*Reader Service No. 101*). Cambridge Technology's model 300B series includes **muscle lever systems** appropriate for studying single fibres up to whole muscles, covering a force range of



Tension products for muscle research.

50–5,000 grams-force. In the length control mode, the levers hold the muscle preparation being tested at a constant length, regardless of the contractile force it generates. The force control mode allows mechanical parameters such as inertia to be cancelled electronically. Cambridge Technology says transient response in either mode of operation is fast, and step length changes of 200 ms are achievable without overshooting or undershooting the required length. Length, velocity and force outputs are provided with the lever systems, which start in price at \$6,500 (US).

DDS Analytical Systems in Denmark has a new kit for the detection of **zeatin plant hormones** which it says is sensitive down to 5 picomoles per gram (*Reader Service No. 102*). The company's kit includes gels and columns for pre-purifica-



The range of products for measuring zeatin plant hormones from DDS Analytical Systems.

tion and immunoaffinity purification, necessary buffers, alkaline phosphatase labelling reagents, zeatin and zeatin riboside standards, and ten pre-coated ELISA plates for performing the assay. The user's manual supplied with the kit describes the principles behind the zeatin hormone test, and spells out the working procedures step-by-step. DDS Analytical Systems says each kit can perform up to 50 analyses.

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For performing the increasingly popular enzymatic DNA amplification techniques, Techne has a new Dri-Block temperature



Techne's temperature cycler for PCR.

cycler with four user-selectable up and down ramp rates (*Reader Service No. 104*). Techne's programmable model PHC-2 thermal cycler has a dummy tube which contains a remote sensor that reads the actual temperature of the sample compartment and prompts the unit to move to the next step in the protocol. Temperatures and times of runs are

stored in memory for subsequent display on the screen or printing to paper. The water-cooled cycler heats samples up to 99 °C at up to 0.8 °C per second, and cools them down to 0 °C at 2.0 °C per second, says Techne. The unit comes in two designs, for holding 0.5 ml or 1.5 ml microtubes.

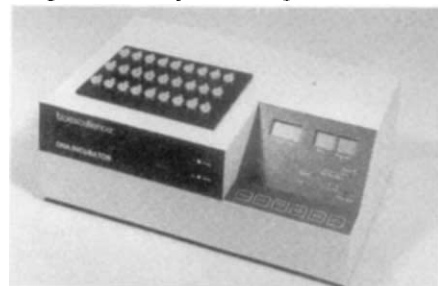
The PREM from LEP Scientific is a microprocessor-controlled **solid-state heating device** that can be used for the polymerase chain reaction for amplifying



LEP Scientific's cycler with 20 programs. specific DNA sequences (*Reader Service No. 105*). LEP Scientific says the PREM heats at a rate of 1 °C per second, with a temperature consistency of better than 0.5 °C across the block. The PREM has 20 preset programs which can be tailored to meet specific research needs. The cycler

holds 41 0.5-ml microtubes, and its solid-state design means no water source is necessary for cooling.

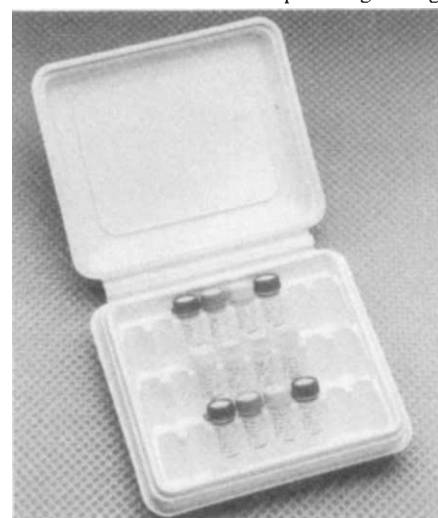
The Dutch company Biores offers a programmable thermal cycler and a reagent kit for performing **DNA amplifi-**



Biores sells a *Taq* DNA polymerase reagent kit with its thermal cycler.

cation reactions (*Reader Service No. 106*). The Biores DNA Incubator can store up to 60 separate programs in memory, with up to 99 cycles per program. The company's accompanying Ampiclone kit is based on thermostable *Taq* DNA polymerase, isolated from the bacteria *Thermus aquaticus*. *Taq* polymerase does not denature at elevated temperatures, eliminating the need to add new enzyme at each amplification cycle.

Taq polymerase is the centrepiece of a new **DNA sequencing kit** from Applied Biosystems, for use with the company's model 370A DNA sequencer (*Reader Service No. 107*). Applied Biosystems designed its *Taq* Polymerase Reagent Kit for the preparation of extension products for automated DNA sequencing using



Taq enzyme for DNA sequencing from Applied Biosystems.

fluorescent-based technology. The company says using *Taq* polymerase in sequencing allows longer runs; more accurate base interpretation, even with GC-rich or palindromic samples; and more uniform band intensities. With less than 1 µg of starting template, Applied Biosystems reports that use of the *Taq* kit allows up to 500 bases per reaction to be

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Reader Service No.10

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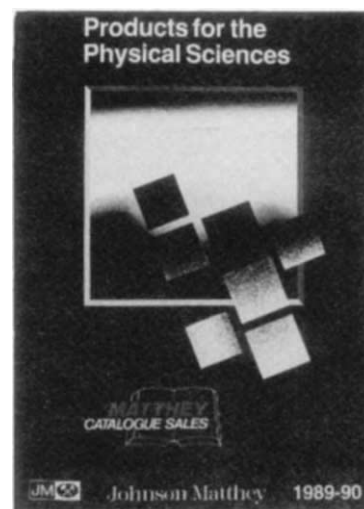
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for the synthesis of high-temperature superconductors in high-pressure oxygen. The furnace can achieve pressures of up to 300 atm at 930 °C, and 200 atm at 980 °C (*Reader Service No. 108*) for the preparation of 1-2-3 and 1-2-4 superconductors, and for oxygen doping. Samples can be changed in roughly ten minutes; the sample compartment measures 1 cm × 5 cm. An electronic pressure gauge with digital readout and a burst disc safety relief valve are provided.

CVC Products, Inc. has a new **thin-film deposition system** for superconductor research — the model SC-4000 (*Reader Service No. 109*). CVC says the small footprint, low-profile system accommodates changing deposition parameters through numerous feedthrough penetrations in the baseplate and flanges in the chamber.

Johnson Matthey's 1989-90 **catalogue of physics products** has just been published (*Reader Service No. 110*). Four thousand products are outlined in the catalogue, including a wide range of pure elements, fabricated metals, inorganic and coordination compounds. The company's REaction rare earth metals and compounds have been widely used in superconductor research laboratories. Johnson



The book from Matthey Catalogue Sales.

Matthey also sells fabricated metal wires, rods and foils; metal single crystals; platinum laboratory apparatus; and homogeneous and heterogeneous chemical catalyst materials. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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Reader Service No.11

The graduate market in the 1990s

Richard Pearson

Supply and demand for graduates in the United Kingdom seem set on convergent paths for the next decade, but graduate unemployment may persist.

THE late 1980s in Britain are proving to be another watershed for higher education and graduate recruiters alike. For the recruiter, continuing economic growth, skill shortages and European integration in 1992 are in prospect. While for higher education, after nearly a decade when its role and finances have been under question by central government, the emphasis has turned to expansion, with the Secretary of State urging that there should be a much larger and more diverse system of higher education as we go into the next century. The aim is to double student numbers over the next 25 years, although no new money is being offered. Ironically this pressure for expansion comes at the very time when the traditional supply of entrants to higher education, the 18-year-olds, goes into steep decline. So, will the students be there, and the jobs they will be expecting?

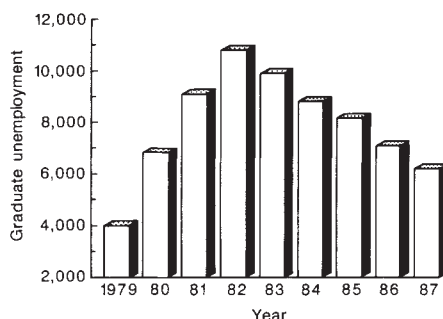
Despite financial cutbacks the output of graduates continued to expand during the 1980s, particularly from the polytechnics and colleges. At the same time the number of graduates going into employment has continued to rise with each year of economic expansion since 1981. In 1986 the numbers being recruited into commercial employment exceeded those going into industry for the first time, reflecting the rise of the service economy. As demand has grown faster than supply in 1988, over half of the graduate recruiters were reporting unfilled vacancies at the end of their graduate recruitment round. These vacancies affected finance and information technology, engineering and teaching, and in particular the public sector.

As employers have faced increasing competition for graduates, differentials in starting salaries have been widening. The median starting salary forecast for a new graduate starting in October 1989 is £10,000, up by 7.5 per cent on the 1988 figure of £9,300. Starting salaries being paid for the 'best' graduates are rising rather faster than the average, and at the top end of the range, salaries of £12,000-£16,000 are being offered by some of the big city accountancy and law firms, the management consultants and some of the oil companies. Firms paying below average include many of the accountancy and legal practices outside London, companies in the leisure and catering section which is relatively new to graduate recruitment, and the health and local authority employers.

But these improving employment prospects have not been reaching all graduates.

While the percentage of graduates unemployed six months after graduation has continued to fall, the number recording themselves as unemployed in 1987 was still higher, at over 6,000, or 6.8 per cent of those graduating, than in 1979 (see figure). On a subject basis we see significant differences with the lowest unemployment being experienced in engineering and technology subjects, averaging under five per cent while in the arts subjects it was over ten per cent.

Graduates are being sought for a wide range of reasons, in some cases for their technical knowledge, in others for their



Graduate unemployment (for both university and polytechnic) in the United Kingdom from 1979 to 1987. These data, and other figures cited in this article, from *The Graduate Labour Market: Prospects for the 1990s* by R. Pearson and G. Pike (IMS, May 1989).

potential and trainability. In other instances graduates are competing directly with other groups in the labour market. The factors determining intake numbers are equally diverse and include corporate growth prospects, technological and organizational change, existing staffing patterns and the availability of alternatives inside and outside the organization.

The major occupational forecasts do, however, suggest that the job opportunities for scientists and engineers will grow by as much as 20 per cent over the decade to 1995, and the number of managers by 12 per cent. At a more detailed level we see more established areas like information technology continuing to expand, as well as professions such as law and accountancy. New sectors such as the leisure industries are following retailing, now among the largest of the graduate recruiters, into graduate recruitment. Many small firms are also following suit while self employment is a growing attraction for graduates. Demand from Europe is likely to grow (*Nature* 338, 526: 1988).

All the evidence suggests that, as long as economic growth continues, job oppor-

tunities for graduates will continue to grow although some of these may be at the expense of other groups in the labour market and not all graduates will be using their skills in their jobs. If the growth rate matches recent trends then the number of vacancies could be 30 per cent higher by the end of the decade, barring any major economic downturn. They will embrace a wide range of opportunities in both small and large, service and manufacturing organizations. Vacancies will be there for both good generalities and specialists.

On the supply side, the numbers graduating are likely to increase by about five per cent over the period to 1992 before falling back until the second half of the 1990s. The precise level of output from 1992 onwards will depend on many factors, not least of which will be competition between employers and higher education for the shrinking supply of 18-year-olds. Also important will be the extent to which higher education will be able to widen its access and attract increasing numbers of 'non-traditional' entrants. The introduction of student loans will be a further important factor affecting demand for places.

Although the numbers graduating are not likely to grow during the second half of the 1980s, the composition of those graduating will change. There will be an increasing share of the graduates coming from the polytechnics and colleges. There will also be proportionately more women, mature students and those with non-traditional qualifications. All of these groups have found it harder to enter the graduate labour market than the traditional young university graduate (*Nature* 337, 100: 1989). There will also be a falling share of engineering and technology graduates, as the continuation of shortages of teachers in mathematics and physics is likely to continue to constrain the supply of students seeking to study engineering and technological subjects.

Unless recruiters adapt to this changing supply and the graduates themselves attune themselves more to labour market needs, we are likely to see a growing segmentation in the graduate labour market in the 1990s with increasing reports of shortages of good graduates coexisting with significant levels of graduate unemployment and under-employment. □

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